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15469

Significance of Antigenic Differences Among Strains of Influenza A Virus in Reinfection of Ferrets.*

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The present paper presents data concerning the significance of the antigenic differences among strains of influenza A virus in the reinfection of ferrets. It has been commonly accepted[1-3] that following an influenza virus infection those animals are, for a time thereafter, "solidly immune" to reinfection with that same strain. However, there are few data showing whether or not during the period of "solid immunity" to one strain, ferrets would fail to show clinical manifestations when reinoculated with strains of virus antigenically related to but different from the strain used for the original infection.

Materials and Methods. Ferrets were inoculated intranasally either with influenza A virus or with control material. A febrile reaction following inoculation was taken as the index of clinical infection. Pools of infected allantoic fluid served as the source of virus and uninfected allantoic fluid was used for the control material. The pools of allantoic fluid were collected before the start of the study and were preserved in the CO_2 ice chest until immediately before use. The ferrets were anesthetized by intraperitoneal injections of nembutal. While anesthetized, the animals were bled from the heart and then were given 1.5 cc of inoculum intranasally. In addition to the bleedings made at the time of inoculation, blood was obtained from each animal during the period of convalescence. The virus-neutralizing antibody content of each serum was determined by means of the mouse protection test; the titers are expressed in terms of the initial dilution of serum which protected 50% of the mice from death.[4] All serums showing protection when diluted 1-1024 or more are recorded as having an antibody titer of 2^{10}.

Five groups of 3 ferrets each were used. Three groups (9 animals) were infected with the CC[5] strain of influenza A virus; the 6 remaining animals which were to serve as controls, were inoculated with uninfected allantoic fluid. Six weeks later all of the animals were reinoculated: 3 infected and 3 control animals received the CC strain of virus, 3 infected and 3 controls received the antigenically related WS[1] strain, and 3 infected animals re-

* From the Strain Study Center, Commission on Influenza, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, United States Army.

1 Smith, W., Andrewes, C. H., and Laidlaw, P. P., *Lancet*, 1933, **2**, 66.

2 Francis, T., Jr., and Stuart-Harris, C. H., *J. Exp. Med.*, 1938, **68**, 813.

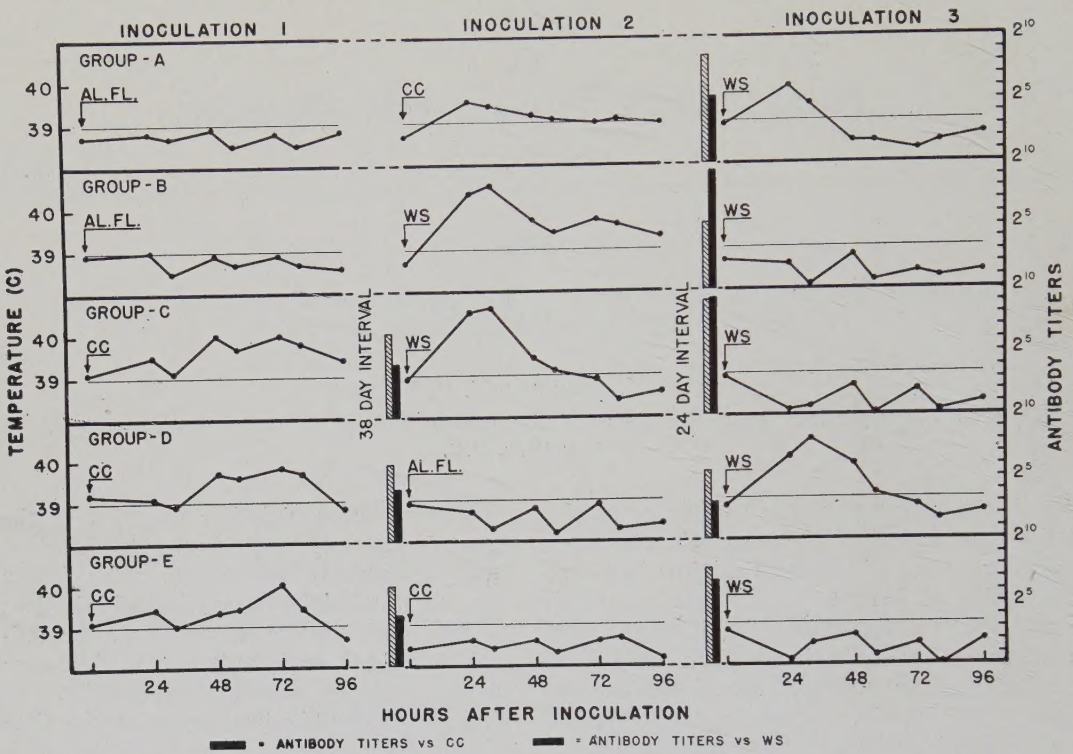
3 Francis, T., Jr., *J. Exp. Med.*, 1939, **69**, 283.

4 Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

5 Magill, T. P., and Sugg, J. Y., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 104.

CHART 1.

Group data. Antibody titers at the time of, and temperature courses following, reinoculation of different strains of influenza virus.



ceived uninfected allantoic fluid. Four weeks after the second inoculation all of the animals were inoculated intranasally with the WS strain.

Results. Temperatures obtained during the 4-day periods following each inoculation, together with antibody titers of the serums obtained at various intervals of time are presented in Chart 1 and in Table I. The results of the neutralization tests with the serums obtained at the time of the first inoculation are omitted because none of those serums possessed demonstrable antibodies against either the CC or the WS strains.

Chart 1 shows graphically, for each of the 5 groups, the average antibody titer at the time of inoculation and the average temperature course following inoculation. Each temperature point and each antibody titer represents the average of data obtained from 3 animals except in the case of Group A, inoculation 3; one of the animals of that group developed an ear infection following inoculation 2 and was discarded.

It is evident (Chart 1) that 4 to 10 weeks following the initial infection ferrets were susceptible to clinical reinfection with influenza virus. The susceptibility to reinfection was influenced by the strain of virus used for reinoculation and by the previous antigenic experience of the animals. Inoculation 2 shows the influence of the strain of virus used for reinoculation upon the febrile responses of ferrets which had had the same previous antigenic experiences. Groups C, D and E all had been initially infected with CC virus. When retested 6 weeks later, the Group C animals, which were inoculated with the antigenically related but different WS strain, gave a pronounced febrile response. On the other hand, the Group E animals, which were retested with CC, showed no more evidence of clinical infection than did the Group D animals which received uninfected allantoic

TABLE I.

Individual Data, Antibody Titers and Febrile Responses to Reinoculation of Different Strains of Influenza Virus.

		Inoculation 1				Inoculation 2						Inoculation 3					
				Antibody titer 2 wks after inoc.		Antibody titer at time of inoc.				Antibody titer 2 wks after inoc.		Antibody titer at time of inoc.				Antibody titer 2 wks after inoc.	
Group	Ferret No.	Inoculum	Febrile response	CC	WS	CC	WS	Inoculum	Febrile response	CC	WS	CC	WS	Inoculum	Febrile response	CC	WS
A	1	Al. Fl.	—	0	0	0	0	CC	+	x*	x	x	x	x	x	x	x
	2	”	—	0	0	0	0	”	+	28.8	24.5	28.8	25.3	WS	+	28.5	27.7
	3	”	—	0	0	0	0	”	+	29.5	24.7	28.5	25.3	”	+	28.5	28.5
B	4	”	—	0	0	0	0	WS	+	26.5	210	25.5	29.5	”	—	26.5	29.8
	5	”	—	0	0	0	0	”	+	25.5	210	25.5	29.3	”	—	25.5	29.3
	6	”	—	0	0	0	0	”	+	25.5	29.5	24.5	29.5	”	—	25.5	210
C	7	CC	+	28.5	22.6	26.5	23.3	”	+	29.5	29.5	28.5	28.3	”	—	28.5	29.3
	8	”	+	28.5	24.3	27.2	24.7	”	+	210	210	29.5	210	”	—	29.8	210
	9	”	+	28.3	24.3	26.5	24.5	”	+	29.5	26.2	29.3	29.3	”	—	29.5	29.3
D	10	”	+	28.5	22.8	27.2	25.3	Al. Fl.	—	26.5	24.3	26.5	24.3	”	+	29.8	210
	11	”	+	28.8	23.5	26.3	23.3	”	—	25.6	22.3	25.3	21.6	”	+	29.5	29.5
	12	”	+	28.5	23.3	24.5	23.3	”	—	24.5	22.6	23.7	21.5	”	+	29.5	210
E	13	”	+	28.5	24.3	26.5	23.3	CC	—	28.5	27.2	26.7	25.5	”	—	28.5	29.3
	14	”	+	28.5	25.3	26.5	24.3	”	—	28.5	26.5	27.2	25.8	”	—	28.8	29.3
	15	”	+	28.5	22.8	26.5	24.5	”	—	28.5	27.5	28.5	27.8	”	—	28.5	28.5

* Ferret No. 1 developed an ear infection following inoculation 2 and was discarded.

fluid. Inoculation 3 shows the influence of the previous antigenic experience upon the responses of ferrets which were inoculated with the same amount of the same virus suspension. Groups A and D, whose previous influenza virus experience consisted of one inoculation with CC (respectively, 4 and 10 weeks previously), had definite febrile responses following inoculation of WS virus. In contrast, the Group E animals whose previous influenza virus experience likewise had been limited to contact with CC but who had received 2 inoculations of that virus (4 and 10 weeks previously) proved to be clinically immune, as did Groups B and C, which had undergone previous infection with WS.

The influence of the strain of virus used for inoculation, and also the influence of the previous antigenic experience were associated with the height of titer of strain-specific antibodies. Comparison of the antibody titers at the time of inoculation with the subsequent temperature courses shows that all groups which gave evidence of clinical infections when retested (Group C, inoculation 2; and Groups A and D, inoculation 3) had lower average titers of antibodies reactive with the strain used for the reinoculation than did any of the groups found to be immune to clinical reinfection (Group E, inoculation 2; and Groups B, C and E, inoculation 3). The lowest titer of the clinically immune groups occurred in Group E, inoculation 3, and was slightly less than 2^7; the highest titer of the clinically susceptible groups occurred in Group A, inoculation 3, and was slightly more than 2^5. Thus, the group data indicate that there was a critical antibody level which separated a state of relative susceptibility from a state of relative resistance, and that the strain-specific titer at the time of inoculation can be taken as an index of that critical antibody level. However, that titer is not necessarily an index of the total amount of antibodies which will be available to combat clinical infection because the speed with which the animal can produce additional antibodies must also be taken into consideration. That the speed of response is influenced by the extent of previous experience of the animal with influenza virus antigens is indicated by the data of the individual animals (Table I).

Table I shows that Ferrets No. 13 and 14 (Group E), at the time of inoculation 3, had WS antibody titers of $2^{5.5}$ and $2^{5.8}$ but showed no signs of infection following inoculation. In contrast, Ferrets No. 2 and 3 (Group A) both had titers of $2^{5.3}$ against WS yet they gave a mild febrile response following inoculation of that strain of virus. The difference between a titer of $2^{5.3}$ on the one hand and of $2^{5.5}$ or $2^{5.8}$ on the other hand probably is insignificant and hardly can explain the difference in the responses of those animals to inoculation of the WS strain of virus. There was, however, a significant difference in the previous influenza virus experience of these ferrets. The more resistant animals (No. 13 and 14) had had the more extensive experience in that they had had 2 inoculations of the CC strain whereas Ferrets No. 2 and 3 had had only one. That the additional experience conditioned the immunological mechanism to respond more quickly is suggested by the fact that 2 weeks subsequent to inoculation Ferrets No. 13 and 14 had significantly higher WS antibody titers than did Ferrets No. 2 and 3. A sufficiently quick antibody response obviously would have enabled the animals to render the virus ineffective before it had multiplied enough to produce symptoms of clinical infection, irrespective of the antibody titer at the time of inoculation.

Discussion. The present investigation dealt with the resistance of ferrets to clinical reinfection with antigenically related but different strains of influenza virus. The main point was that 4 to 10 weeks following an initial infection the animals, in all cases, were immune to reinfection with the same strain, but were susceptible to reinfection with a strain of virus which was antigenically related to but different from the strain used for the original infection. It would seem, therefore, that the antigenic differences which are known to exist among the A strains of influenza virus are of sufficient magnitude to be of practical importance in problems dealing with immunity to influenza. For example, an immunization procedure might be adequate to produce a high degree of resistance to infection with some strains of influenza virus but afford little protection against infection with

other, although antigenically related strains.

The results indicate that for a constant amount of virus there was a critical level of specific antibodies which separated a state of relative resistance from a state of relative susceptibility. However, the height of titer at the time of inoculation was not always an index of the resistance of the animal to clinical infection. When ferrets which had approximately the same titers of specific antibodies were inoculated with the same amount of the same virus suspension, the animals which had had the more extensive previous experience with influenza virus strains were likewise the more resistant to clinical infection. The more extensive experience apparently endowed those animals with a more active immunological mechanism which enabled them to mobilize more quickly a higher concentration of strain-specific antibodies. In so far as protection against clinical infection is concerned the protective effect of antibodies is not limited to the quantity present at the time of inoculation but is dependent upon the amount made available before the infecting agent has multiplied sufficiently to evoke clinical manifestations.

Summary. Four to 10 weeks following an initial infection with influenza A virus, ferrets were found to be immune to clinical reinfection with that same strain of virus but were susceptible to reinfection with a strain of virus which was antigenically related to but different from the strain used for the original infection.

15470 P

Filamentous Forms of Newcastle Virus.

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Relatively few of the animal viruses have been purified and studied under the electron microscope.[1] This preliminary report deals with the causative agent of Newcastle disease of chickens.

Four strains of virus have been studied, 2 isolated in New Jersey, 2 in California. Strain B was isolated from a field case by Dr. F. R. Beaudette and furnished to us in the 1st embryo passage. Strain W was isolated by us from an outbreak of the disease in a flock of White Leghorns at Bound Brook, N. J. Strain Nap is a laboratory strain isolated by Dr. J. R. Beach in California. These 3 strains are identified as Newcastle virus by their behavior in the developing chick embryo, which they kill with specific hemorrhagic lesions of the brain, feather follicles, and entire embryo in 48 to 84 hours,[2] by their ability to agglutinate chicken red blood cells, by the neutralization of these effects by specific antisera against known strains, and by the reproduction of the natural disease in chickens.[3,4] Strain Cg179, also furnished by Dr. Beach, is highly virulent for chickens and is specifically neutralized by antisera in the embryo.

All four of these strains when partially purified from allantoic fluid by 2 cycles of differential ultracentrifugation show filamentous forms which predominate on the screen of the electron microscope. (Fig. 1 and 2); the phagelike structure is not a constant feature of our preparations, but at no time have we failed to demonstrate filamentous forms. That these forms represent active virus is indicated by their presence in concentrates of the allantoic fluid as early as 24 hours after inoculation until 48 to 72 hours when death of the embryo occurs; by their presence at tem-

1 Wyckoff, R. W. G., *Science*, 1946, **104**, 21.

2 Burnet, F. M., *Aust. J. Exp. Biol. and Med. Sci.*, 1942, **20**, 81.

3 Beaudette, F. R., *Cornell Vet.*, 1946, **36**, 105.

4 Beach, J. R., *J. Am. Vet. Med. Assn.*, 1946, **108**, 372.

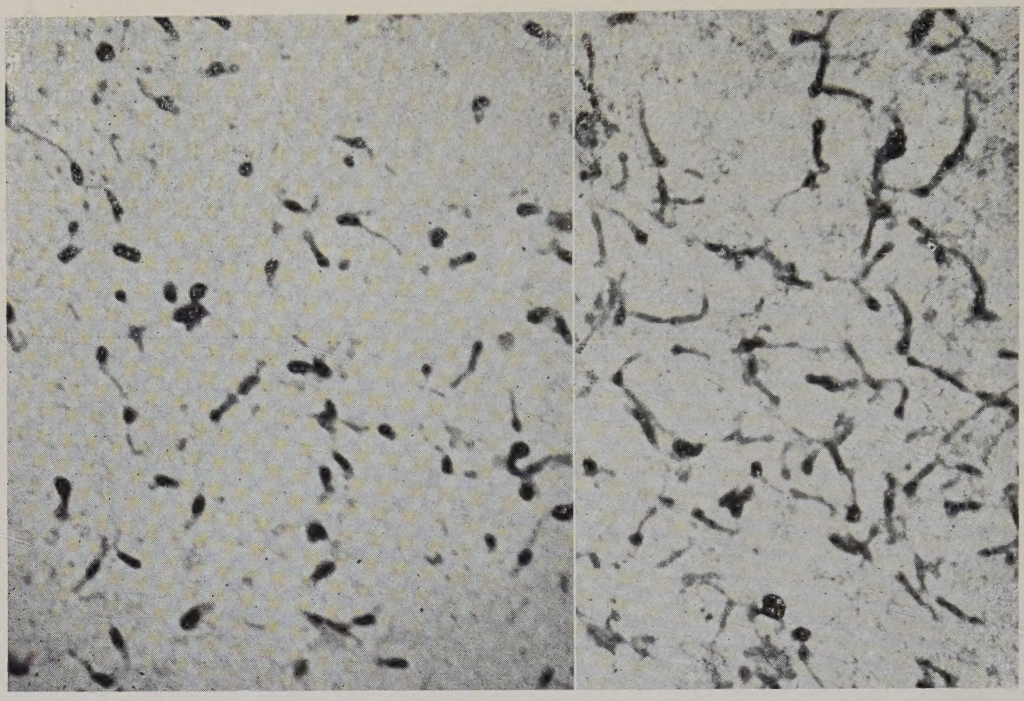

Fig. 1. (Left).
Electron microscope photograph of concentrate of strain B Newcastle virus. 0.01% formaldehyde 2 days. × 17,200.

Fig. 2. (Right).
Concentrate prepared immediately after centrifugation and resuspension in buffered saline. × 17,200.

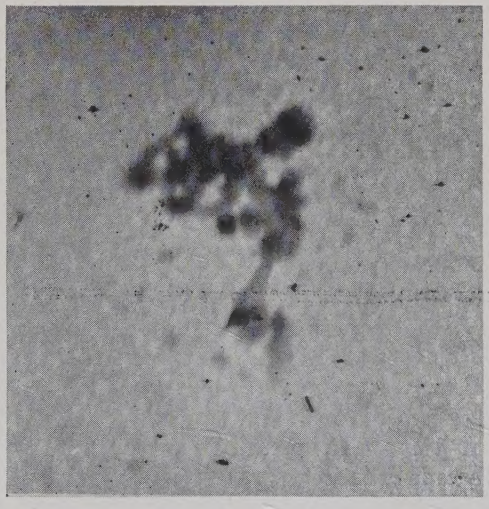

FIG. 3.
Effect of immune sera. Screen prepared 2 hr after the addition of antisera. Control sera failed to agglutinate virus. × 17,200.

peratures of incubation of the inoculated embryo from 35° to 39°C; by the stability of the virus during centrifugation (see Table I); and by the facts that calculations of size and weight of the virus by electronscope micrography and by light scattering measurements[5] yield a size of about 115 mμ and a molecular weight of 450 million,* which on the basis of demonstrated infectivity for the embryo (Table I) indicates that 10 or less particles are able to infect the embryo; that concentrates of the virus may be specifically precipitated by antisera and electron microscope pictures show the filamentous forms to be clumped by these antisera; and that the sharpness of outline of these forms is gradually lost as the suspension stands and as chick embryo in-

5 Oster, G., *Science*, 1946, **103**, 306.

* We are indebted to Dr. R. M. Herriott for these determinations.

TABLE I.
Purification of Newcastle Virus by Ultracentrifugation.

	Original material			1st supernatant		
	Titer by			Titer by		
Exp. No.	Embryo inoculation	Chicken r.b.c. agglutination	N mg/cc	Embryo inoculation	Chicken r.b.c. agglutination	N mg/cc
1	$10^{9.5}$	1/4000	.48	$10^{6.0}$	1/20	.47
2	$10^{8.5}$	1/800		$10^{6.7}$	1/10	
3	$10^{8.7}$	1/400		$10^{6.3}$	1/10	
4	$10^{8.6}$	1/1600			Undiluted	
5	$10^{8.3}$	1/3200			1/16	
6	$10^{8.7}$	1/800			Undiluted	

	Final concentrate			
	Titer by			
Exp. No.	Embryo inoculation	Chicken r.b.c. agglutination	N mg/cc	Titer/ g protein
1	$10^{10.3}$	1/4000	.014	$10^{15.4}$
2	$10^{8.7}$	1/400	.007	$10^{14.1}$
3	$10^{9.5}$	1/400	.01	$10^{14.8}$
4	$10^{9.0}$	1/800	.008	$10^{14.4}$
5	$10^{9.0}$	1/3200	.004	$10^{14.7}$
6	$10^{8.6}$	1/800	.0036	$10^{14.4}$
			Average	$10^{14.6}$

Virus was titered by calculating the 50% end point mortality after inoculation of 10-11 day chorioallantoic membranes. Since by Avagadro's law a gram of a substance with a molecular weight of 450 million would contain 1.3×10^{15} particles, and since the average preparation here titered to $10^{14.6}$ or 4×10^{14}, there is a three-fold difference between actual and possible end points. The crudeness of the method of titration does not warrant further calculation.

fectivity is lost.

We have not seen these forms in preparations of allantoic fluid containing the same concentration of virus before purification and resuspension in phosphate buffered saline solution. Whether this is due to a masking of form by the allantoic fluid in which the virus exists or whether it is due to a change of shape on transfer into a new medium is undetermined as yet.

Summary. Studies on purified preparations of Newcastle virus of chickens demonstrate that this virus may be filamentous or stringy in form, sometimes with a large head, approaching the tailed forms of the bacteriophages or bacterial viruses.

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Cultivation of the Murine SK Strain of Poliomyelitis Virus in Developing Eggs.*

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Dunham and Parker,[1] Gard,[2] and recently Riordan and Sá-Fleitas[3] have reported the cultivation of Theiler's mouse encephalomyelitis virus in developing eggs. Similar attempts to propagate strains of human poliomyelitis virus in eggs have failed;[4–7] a doubtful result, in which one monkey inoculated with egg passage material developed paralysis long after the usual incubation period, has been reported by Gard.[8]

The present report deals with the propagation in fertile eggs of the murine SK strain of poliomyelitis virus described by Jungeblut and Sanders[9] as a strain they had adapted to mice from a monkey passage virus, originally isolated by Trask, Vignec, and Paul[10] from the stools of a child with nonparalytic poliomyelitis. Unlike the mouse-adapted Lansing strain,[11] this strain no longer readily induces typical poliomyelitis in monkeys.[12] It is distinctive also in that it attains high concentrations in mice and is infectious for them by a variety of routes.

Sanders and Jungeblut[13] in 1942 reported the cultivation of this strain in serum ultrafiltrate containing embryonic mouse tissue and made observations on the conditions which favor its growth. The amount of growth was influenced by the relative amount of nervous tissue represented in the cultures and was greatest in the presence of brain tissue. No growth was obtained when embryonic chick tissue was used in place of mouse tissue, and none was obtained in embryonated eggs. Schultz and Irwin[14] were able to confirm the multiplication of this strain in the presence of minced embryo mouse brain tissue and serum ultrafiltrate. The virus was carried through 14 culture passages. It was also carried through a similar number of passages in which minced mouse embryo intestines was used in place of brain tissue. The mouse-adapted Lansing strain of poliomyelitis virus failed to show growth under similar conditions. Chick embryo tissue was not tried.

The present observations were made on fertile eggs incubated for 9 days at 100°F in an egg incubator. The initial inoculum consisted of 0.1 ml of a 10^{-5} dilution of filtered mouse brain suspension, prepared as follows: A 10% mouse brain suspension in broth was filtered through a coarse Mandler candle and diluted serially, in 10-fold dilutions with broth, through to a final dilution of 10^{-7}, counting the unfiltered dilution as 10^{-1}. The 10^{-5} dilution induced infection in 3 out of 3 mice inoculated with 0.025 ml of the dilution intracranially; the 10^{-6} dilution induced infection in 2 out of 4 mice, while 10^{-7} dilution failed to induce infection in all of 5 mice so inoculated.

* Supported by the Howard Frost Poliomyelitis Research Fund.

1 Dunham, W. B., and Parker, Sue, *J. Bact.*, 1943, **45**, 80.

2 Gard, S., *Acta Med. Scand.* (Suppl), 1943, 143.

3 Riordan, J. T., and Sá-Fleitas, M. J., *Science*, 1946, **103**, 499.

4 Burnet, F. M., *Med. J. Australia*, 1935, **1**, 46.

5 Kast, Clara, and Kolmer, J. A., *J. Infect. Dis.*, 1937, **61**, 60.

6 Stimpert, F. D., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 483.

7 Gavrilov, M. W., and Fester, A., *Arch. f. Ges. Virusforsch.*, 1940, **1**, 404.

8 Gard, S., *Nature*, London, 1943, **152**, 660.

9 Jungeblut, C. W., and Sanders, M., *J. Exp. Med.*, 1940, **72**, 407.

10 Trask, J. D., Vignec, A. J., and Paul, J. R., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 147; 1939, **41**, 241; *J. A. M. A.*, 1938, **111**, 6.

11 Armstrong, C., *Pub. Health Rep.*, 1939, **54**, 1719.

12 Jungeblut, C. W., Sanders, M., and Feiner, R. R., *J. Exp. Med.*, 1942, **75**, 611.

13 Sanders, M., and Jungeblut, C. W., *J. Exp. Med.*, 1942, **75**, 631.

14 Schultz, E. W., and Irwin, Elizabeth A., unpublished observations.

TABLE I.
Incidence of Deaths in Embryos and Results of Tissue Assays for Virus in Individual Egg Passages.

Passage No.	Route of inoculation	Incidence of deaths in embryos	Tissues assayed	M.I.D.¶ of virus per g tissue
3	C-A*	11/12§		
	Y-S†		Heads	4×10^5
	I-E‡			
5	C-A	5/5	C-A	4×10^5
			Heads	4×10^5
			Viscera	4×10^6
	Y-S	4/5	C-A	4×10^4
			Heads	4×10^6
			Viscera	4×10^7
	I-E	3/4	Brain	4×10^5
6	C-A	5/6	Heads	4×10^6
	Y-S	5/6	Viscera	4×10^7
7	C-A	5/6	Heads	4×10^6
	Y-S	3/4	Viscera	4×10^6
8	C-A	5/6	Heads	4×10^6
	Y-S	6/6	Viscera	4×10^6
10‖	C-A	2/6	Heads	4×10^6
	Y-S	3/6	Viscera	4×10^6
11	C-A	5/6	Heads	4×10^7
12	C-A	11/18	"	4×10^6
13‖	C-A	5/13	"	4×10^5
14	C-A	14/19	"	4×10^5

* Chorioallantoic route.
† Yolk-sac route.
‡ Intra-embryonic route.
§ Denominator = total number of eggs inoculated; numerator, number of deaths in embryos between the 2nd and 5th days.
‖ Inoculated eggs incubated at 37°C, instead of 35°C.
¶ M.I.D. = minimal infectious doses.

During the first 5 passages, groups of eggs were inoculated by one of 3 different routes: (a) chorioallantois according to Burnet,[15] (b) into the yolk sac according to Cox,[16] and (c) intraembryonically according to Elmendorf and Smith.[17] After inoculation, the eggs were incubated at 35°C and were candled daily over a period of 5 days to determine the viability of the embryos. A high per cent became nonviable. A few which became nonviable within 24 hours were discarded. Eggs in which death of the embryo occurred between the 2nd and 5th day were stored in a refrigerator at 4°C. On the 5th day all of the embryos were harvested, including those still viable; the material was pooled and prepared for passage to the next group of eggs. In preparing the material for the next passage, the heads and necks of the individual embryos were pooled and ground in a mortar to a uniform paste. This was emulsified in broth to make a 10% suspension. After centrifugation at 2000 r.p.m. for 10 minutes the supernatant was filtered through a coarse Mandler candle. One-tenth ml of this filtrate served as the inoculum for the next passage.

15 Burnet, F. M., Medical Research Council, Special Reports Series No. 220, 1936, pp. 1-58.

16 Cox, H. R., *U. S. Pub. Health Rep.*, 1938, **53**, 2241.

17 Elmendorf, J. E., and Smith, H. H., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 171.

Titrations were carried out on filtrates from each passage.

In assaying the virus content of the embryos from the 5th passage, eggs were separated on the basis of the routes by which the inoculations were made and different portions of the embryos in each group were titrated separately for virus content. These portions included pools of the chorioallantois, pools of the heads of embryos, and pools of the abdominal viscera (kidneys, liver, spleen, stomach and intestine). In those inoculated by the intraembryonic method, pooled brains only were titrated for virus.

After the 5th passage the intraembryonic method was abandoned, and the passages made by the 2 remaining routes were carried independently of each other. In the passages which were made by the chorioallantoic route, the material for the next inoculation was always obtained from the pooled heads of embryos harvested from the previous passage; in those made by the yolk sac route, the material inoculated was always harvested from the pooled viscera of embryos previously infected by the yolk sac route. The purpose of these 2 separate lines of passage was to determine whether the relative difference in the amount of nervous tissue represented would influence appreciably the amount of virus produced.

It has been stated that a high per cent (about 80%) of the embryos died between the 2nd and 5th days. These deaths occurred from the first passage onward. No distinctive gross or microscopic lesions in either the chorioallantois or the embryos have thus far been identified, but this is under further study. The incidence of deaths in embryos in eggs incubated at 37°C was lower (40%) than that in eggs incubated at 35°C.

Our observations are summarized in Table I. Results on certain of the passages have been omitted to save space. These are in agreement with those given. All of the titrations were carried out by inoculating the individual dilutions of the passage material intracranially into groups of 3 to 5 white mice, each with 0.025 ml of the dilution, and 50% infection in the individual groups was counted as the end point.

Neutralization tests against anti-SK serum, prepared before these studies were initiated, were carried out with virus from the 10th egg passage. The filtrate used in the tests titered 10^{-5}, based on 50% infection. It was employed in a dilution of 10^{-4} and 0.5 ml of this dilution was treated with an equal volume of the individual serum dilutions. Complete neutralization was obtained in serum dilutions up to and including 1:512, the highest serum dilution tested. Theiler's GD-VII strain of mouse encephalomyelitis virus was not neutralized by the serum.

Summary. The murine SK strain of poliomyelitis virus was carried through 14 passages on developing eggs without evident diminution of virus content. Its multiplication was associated with a high incidence of deaths in the embryos. The incidence of deaths was influenced by the temperature at which the eggs were incubated after inoculation.†

† Since this manuscript was submitted, we have made observations which indicate that the Lansing strain also, may be cultivated under these conditions.

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Alopecia in Rats Fed Certain Soybean Oil Meal Rations.*

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It is generally believed that the rat does not require dietary sources of inositol and biotin when purified rations are fed. However, certain dietary constituents may in some

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TABLE I.
Effect of Biotin and Inositol on Hair Loss (Experiment I).

Lot No.	Ration	Total No. of rats	No. of rats showing hair loss	Avg wt in g after 6 wks (initial wt, 40 g)
1	Soybean oil meal basal	20	13	135
2	S.O.M. basal + 0.1% inositol	32	23	141
3	S.O.M. basal + 0.1% inositol + 4 μg biotin daily (oral)	32	0*	141
4	S.O.M. basal + 0.1% inositol + 4 μg biotin daily (inj)	24	0*	144
5	S.O.M. basal + 0.3% inositol	6	0	132
6	Raw soybean oil meal basal	22	15	94
7	Raw S.O.M. basal + 1 μg biotin daily (oral)	14	2	104
8	Raw S.O.M. basal + 0.3% inositol	8	0	91

* In each of these groups there was one rat which showed early signs of hair loss but spontaneous cure followed.

manner bring out a need for one or both of these vitamins. It is well known that the inclusion of a sulfonamide drug in a purified ration may give rise to biotin and inositol deficiencies.[1,2] The drug probably acts by increasing the demands of the organism beyond its synthetic capacities for the factors or by inhibiting intestinal organisms which ordinarily act as a source of these factors.[3,4] An inositol deficiency has also been produced by feeding a ration composed largely of corn, soybean oil meal and alfalfa.[5] It was suggested that the need for inositol when this natural ration was fed was the result of a vitamin imbalance or of some change in the intestinal flora. A biotin deficiency will also result if a ration containing raw egg white is fed.[6,7] The avidin of the egg white combines with the biotin in the intestinal tract thus forming a complex which is unavailable to the rat.[8]

We have observed an alopecia in rats fed certain soybean oil meal rations which was prevented by supplements of biotin and/or inositol. The condition did not develop when the rations were supplemented with cystine or methionine.

Experimental. Weanling male and female rats of Sprague-Dawley breeding were used in these studies. The soybean oil meal basal ration was composed of soybean oil meal (expeller processed, commercial) 34.0 g, sucrose 57.5 g, salts IV 4.0 g,[9] corn oil 4.0 g, choline chloride 0.3 g, niacin 2.5 mg, calcium pantothenate 1.0 mg, pyridoxine 0.3 mg, thiamine hydrochloride 0.3 mg, riboflavin 0.3 mg, and 2-methyl-1, 4-naphthoquinone 0.1 mg, per 100 g ration. The raw soybean oil meal basal ration differed from this ration in that unheated solvent extracted flakes replaced the soybean oil meal. Two drops of halibut oil were given weekly. Oral biotin supplements were given by dropper, and injected biotin supplements were injected intradermally in saline solution.

Rations were mixed every 2 weeks and were stored at refrigerator temperatures to avoid possible development of rancidity. Rations and distilled water were given daily to insure *ad libitum* consumption.

Results. Experiment I. Table I summarizes growth data and the incidence of alopecia in rats fed the soybean oil meal rations. Only small differences in growth rate

1 Nielsen, E., and Elvehjem, C. A., *J. Biol. Chem.*, 1942, **145**, 713.

2 Nielsen, E., and Black, A., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 14.

3 Woolley, D. W., *J. Nutrition*, 1944, **28**, 305.

4 Nielsen, E., and Black, A., *J. Nutrition*, 1944, **28**, 203.

5 Cunha, T. J., Kirkwood, S., Phillips, P. H., and Bohstedt, G., PROC. SOC. EXP. BIOL. AND MED., 1942, **54**, 236.

6 Boas, M. A., *Biochem. J.*, 1927, **21**, 712.

7 Nielsen, E., and Elvehjem, C. A., *J. Biol. Chem.*, 1942, **144**, 405.

8 Eakin, R. E., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, 1940, **136**, 801.

9 Phillips, P. H., and Hart, E. B., *J. Biol. Chem.*, 1935, **109**, 657.

TABLE II.
Effect of Cystine and Methionine on Growth and Hair Loss (Experiment II).

Lot No.	Ration	Total No. of rats	No. of rats showing hair loss	Avg wt in g after 6 wk (initial wt, 40 g)
1	Soybean oil meal basal + 0.1% inositol	8	7	132
2	S.O.M. basal + 0.1% inositol + 0.2% *l*-cystine	8	0*	143
3	Raw soybean oil meal basal	16	10	87
4	Raw S.O.M. basal + 0.4% *l*-cystine	8	0*	126
5	Raw S.O.M. basal + 0.4% *dl*-methionine	8	0*	134

* In each of these groups there was one rat which showed early signs of hair loss but spontaneous cure followed.

were observed in the rats receiving the soybean oil meal basal ration and supplemented rations (lots 1-5)). However, 13 of 20 rats receiving the soybean oil meal basal ration (lot 1) developed a marked alopecia. The condition was first observed during the 4th week of the experiment and in all cases was still evident at the end of the 12-week experimental period. The hair loss started in the dorsal region of the head and proceeded bilaterally along the sides to the tail region. All the hair loss reported herein followed this general pattern and persisted throughout the experimental period unless otherwise indicated. The alopecia was not prevented by supplementation with 0.1% inositol (lot 2). The condition was prevented when the basal ration was supplemented with 0.3% inositol (lot 5), or a combination of 0.1% inositol and a daily biotin supplement of 4μg. Oral administration or intradermal injections of biotin were equally effective (lots 3 and 4).

The growth rate of rats fed the raw soybean oil meal basal ration (lot 6) was less than the growth rate of rats receiving the heated commercial soybean oil meal basal ration (lot 1). A slight stimulatory effect on growth was observed when biotin was added to the raw soybean oil meal basal ration (lots 6 and 7). Hair loss similar to that observed in lots 1 and 2 was also noticed in lot 6 when the raw soybean oil meal basal ration was fed. Fifteen of 22 rats in the later group exhibited the characteristic symptoms. Supplementation of this ration with biotin (1 μg per rat per day, lot 7) reduced the incidence of hair loss. Only 2 rats in a group of 14 showed any signs of alopecia. When the raw soybean oil meal basal ration was supplemented with 0.3% inositol (lot 8) no hair loss occurred.

Experiment II. The effects of adding cystine and methionine to the soybean oil meal rations are summarized in Table II. The characteristic hair loss was again observed when the soybean oil meal basal ration was supplemented with 0.1% inositol (lot 1). It also developed when the raw soybean oil meal ration (lot 3) was fed. However, when these rations were supplemented with cystine or methionine no permanent alopecia was noticed. In each of these supplemented groups (lots 2, 4, 5) there was one rat that showed early signs of hair loss which cleared up spontaneously.

Supplementation of the rations with cystine and methionine also resulted in a marked growth stimulus, especially in the case of the raw soybean oil meal rations.

Discussion. Apparently, soybean oil meal in some manner increases the dietary requirement of biotin and/or inositol. This effect may be due to alteration in the intestinal flora, to some absorption disturbance, to a vitamin imbalance or to the action of antivitamins which may be present in soybean oil meal. It is possible that only one of these vitamins is the limiting factor and that the second factor acts only in the formation of the first. It is also possible that both of these factors function in the formation of a third factor which is really the necessary factor. It is interesting that the condition does not develop when the soybean oil meal rations were supplemented with cystine or methionine. It seems possible that inositol

and/or biotin may be closely related to cystine and methionine in normal hair formation.

Another type of hair loss, referred to as the "spectacle eye" condition, has been associated with a biotin deficiency by Neilsen and Elvehjem[10] and to an inositol deficiency by Pavcek and Baum.[11] It seems possible that these two factors may be closely related.

The commercial soybean oil meal rations supported better growth than did the raw soybean oil meal rations. Cystine and methionine supplements stimulated growth especially when added to the raw soybean oil meal rations. These findings support the observations reported by earlier investigators.[12,13] Biotin supplements had a slight stimulatory effect on growth when added to the raw soybean oil meal basal ration. Cunha[14] reported earlier that biotin supplements stimulated growth in rats fed a diet consisting largely of corn, soybean oil meal and alfalfa.

Further investigations now in progress suggest that the situation may become more complicated under certain conditions. While low levels (1-4 μg) of biotin have been shown to prevent the loss of hair, higher levels (12 μg) of this vitamin may actually accentuate the condition. The hair loss resulting from feeding such a high level of biotin was prevented by supplementation with adequate inositol.

Summary. Rats fed certain soybean oil meal rations developed a characteristic hair loss which was prevented by supplementation of inositol and/or biotin. The condition did not develop if the rations contained added cystine or methionine. Inositol supplementation resulted in no marked changes in rate of growth. However, supplements of cystine and methionine markedly increased the rate of growth when added to the raw soybean oil meal basal ration. Biotin supplements had a slight stimulatory effect on growth when added to the same ration. It is believed that soybean oil meal in some manner increases the dietary requirement for biotin and/or inositol. Possible mechanisms for such action may be an alteration of the intestinal flora, some absorptive disturbance, a vitamin imbalance or the presence of antivitamins in the soybean oil meal.

We are indebted to Merck and Co., Rahway, N.J., for synthetic vitamins; and to Abbott Laboratories, North Chicago, Ill., for halibut liver oil.

10 Nielsen, E., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 349.

11 Pavcek, P. L., and Baum, H. M., *Science*, 1941, **93**, 502.

12 Hayward, J. W., Steenbock, H., and Bohstedt, G., *J. Nutrition*, 1936, **11**, 219.

13 Hayward, J. W., and Hafner, F. H., *Poultry Sci.*, 1941, **20**, 139.

14 Cunha, T. J., Ph.D. Thesis, University of Wisconsin, 1944.

15473

Effect of Low Protein Diets upon Creatine Excretion of the Rat.

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In a previous study[1] qualitative tests upon the urine of rats after a period on a low protein and low choline intake suggested an increased excretion of creatine by these animals. Roberts and Eckstein[2] have shown that the creatine content of the gastrocnemius muscle of young rats is not lowered when a diet deficient in choline and methionine is fed for 3 weeks. A similar finding in chicks was reported by Almquist and coworkers[3] who found a deficiency in dietary choline did not appreciably diminish the muscle creatine.

1 Tidwell, H. C., and Treadwell, C. R., *J. Biol. Chem.*, 1946, **162**, 155.

2 Roberts, E., and Eckstein, H. C., *J. Biol. Chem.*, 1944, **154**, 377.

3 Almquist, H. J., Kratzer, F. H., and Mecchi, E., *J. Biol. Chem.*, 1943, **148**, 17.

TABLE I.
Weight changes and Creatine and Creatinine Excretion of Rats on 5% (Diet 1) and 25% (Diet 2) Protein Diets.

Diet No.		Body wt in grams and % of change. Control	4th da.	8th da.	12th da.	16th da.	20th da.
1	(g)	236	231	226	224	225	226
	(%)	—0.0	—2.1	—4.2	—5.1	—4.7	—4.2
2	(g)	232	239	245	255	260	266
	(%)	+0.0	+3.0	+5.6	+9.9	+12.1	+14.7
		Creatinine excreted—mg/100 g rat.*					
1		3.69 ±.10	3.82 ±.10	3.50 ±.16	3.53 ±.12	3.71 ±.08	3.73 ±.06
2		3.48 ±.12	3.45 ±.10	3.48 ±.10	3.49 ±.08	3.77 ±.08	3.66 ±.09
		Creatine excreted—mg/100 g rat.*					
1		0.61 ±.15	0.40 ±.05	1.18 ±.18	1.32 ±.48	1.19 ±.32	0.62 ±.09
2		0.56 ±.12	0.50 ±.06	0.51 ±.13	0.71 ±.14	0.59 ±.14	0.57 ±.04

* Including the standard error of the mean calculated as follows:

$$\sqrt{\Sigma d^2 / n - 1} / \sqrt{n}$$

These observations along with those that indicate that the physiologically labile methyl groups are preferentially used for growth rather than lipotropism[4] suggest no sparing of these groups as regards the synthesis of creatine.

This study was designed to obtain more information regarding total creatine formation in these rats as measured by its excretion rather than the creatine content of an isolated tissue. Such information would indicate whether the labile methyl groups are conserved in the body when there is an inadequate supply for lipotropic action, and whether the needs for creatine formation take precedence over the needs for lipotropism.

Methods. After 3 weeks on the stock diet (Rockland rat diet), 20 male white rats were divided into 2 equal groups, each averaging about 235 g. They were maintained for 21 days on the special diets containing 5 or 25% casein, 40% Crisco, 5% salt mixture,[5] 2% Cellu flour, and sufficient starch to complete the diet. In addition each rat received one yeast tablet (400 mg) and 2 drops of cod liver oil daily. This diet has been used repeatedly in this laboratory for the production of fatty livers. It seemed possible that the methyl groups required for the synthesis of creatine and choline might be conserved during the acute need for lipotropic substances. If so, this should be reflected in the creatine and creatinine excretion.

The urine was collected under light mineral oil over 24-hour periods while on the stock diet and every 4th day through the 21-day period on the special diets. Intraperitoneal injections of 5 cc of 0.9% saline were given each rat twice daily on the days the urine was collected so as to obtain more suitable urine volumes. The creatine and the creatinine excreted were determined according to the method of Folin[6] with color intensities measured photoelectrically.

Results. More creatinine was excreted per 100 g body weight on the 4th day by the animals receiving the low protein diet and thereafter the amount excreted remained the same during the test period for the animals

[4] a. Treadwell, C. R., Groothuis, M., and Eckstein, H. C., *J. Biol. Chem.*, 1941, **142**, 653; b. Treadwell, C. R., Tidwell, H. C., and Gast, J. H., *J. Biol. Chem.*, 1944, **156**, 237.

[5] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, **37**, 572.

[6] Folin, O., *J. Biol. Chem.*, 1914, **17**, 469.

on both diets (Table I). A significantly greater* amount of creatine was excreted by the animals on the diets deficient in methionine and choline on the 8th and possibly the 12th days only. This increased excretion of creatinine at the start, and of the creatine later, appeared to be associated with the time when the animals were losing weight. Generally an increased creatine excretion was associated with a weight loss in the individual animals whenever it occurred during the experimental period. The differences in creatine and creatinine excreted by the 2 groups were not significant during the latter part of the period when the animals on the low protein diet maintained their weight or gained slightly.

The excretion of similar or greater amounts of creatine or creatinine by the animals on the diet deficient in methionine and choline for lipotropism, gives no indication of a deficiency of methyl groups for creatine formation. This again suggests that there is a preferential use of labile methyl groups for creatine formation associated with the demand for growth. Apparently there is no conservation of the deficient supply of methyl groups during the time fatty livers are developing in these animals, but actually a wastage due to the increased creatine or creatinine excreted when the fed animals are losing weight. The general constancy of the creatinine excretion is in line with the finding of Borsook and Dubnoff[7] that under physiological conditions the phosphocreatine spontaneously yields 2% of free creatinine and that its excretion does not characterize any active process in the tissues. The increased creatinine excretion during the early part of the test period was probably due to a readjustment of the body fluids to the new dietary conditions. The loss of weight involving muscle tissue would explain the increased creatinuria.

Summary. No diminution in the formation of creatine, as measured by its urinary excretion, was found in animals on a diet deficient in labile methyl groups. The available methionine was preferentially used for growth and creatine formation. The increased excretion of creatine by these animals, apparently associated with weight loss, causes a waste of needed methyl groups instead of their conservation.

* Apparent differences were analyzed for significance by the *t* method of Fisher, R. A., *Statistical Methods for Research Workers*, 7th edition, Edinburgh, 1938. Only those showing a *P* value of 0.01 or less were considered significant.

Grateful acknowledgment is made to Dr. C. R. Treadwell for his generous advice.

7 Borsook, H., and Dubnoff, J. W., *Annual Review of Biochemistry*, 1943, **12**, 187.

15474

Urinary Secretion of Acetone Bodies in Diabetic Ketosis.

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It is generally recognized[1] that the urine from a patient with diabetic coma, occasionally, may give a negative test for diacetic acid with ferric chloride, and only a mild reaction for acetone bodies with nitroprusside. The studies of Widmark,[2] and Briggs and Shaffer[3] indicated that acetone passes into the urine and expired air by the simple process of diffusion. If this is true, then the concentrations of this fraction of the acetone bodies in the blood and urine should always

1 Joslin, E. P., Root, H. W., White, Priscilla, and Marble, A., *The Treatment of Diabetes Mellitus*, seventh ed., Lea and Febiger Co., Philadelphia, 1940.

2 Widmark, E. M. P., *Biochem. J.*, 1920, **14**, 364.

3 Briggs, A. P., and Shaffer, P. A., *J. Biol. Chem.*, 1921, **48**, 413.

TABLE I.
Concentrations of Acetone Bodies in Blood and Urine from Patients with Diabetic Ketosis.

	Blood Quantitative mg %			Urine Quantitative mg %		Urine Qualitative		
Case No.	Acetone	Total acetone bodies	Glucose	Acetone	Total acetone bodies	$FeCl_3$	Nitro-prusside	Sugar (Benedict's)
1	23.7	90.6	889	22.2	319.2	+++	++++	++++
2	21.5	94.0	507	27.8	436.5	+++	++++	++++
3	25.6	114.0	680	26.2	63.0	Neg.	++	++++
4	29.4	91.0	478	29.7	86.0	Neg.	++	++++
5	33.2	111.0	641	37.6	741.0	++++	++++	++++
6	5.8	28.1	358	5.1	63.3	+	+++	++++
7	6.5	29.6	484	7.7	72.1	++	+++	++++
8	3.0	9.2	390	2.8	36.4	Neg.	+++	++++
9	1.9	4.9	306	2.0	15.2	Neg.	+	++++
10	1.2	5.2	320	1.2	17.8	Neg.	++	++++
11	1.8	3.9	310	2.2	14.8	Neg.	++	+++
12	1.2	3.0	253	1.4	10.6	Neg.	+	+++

be similar; and this relationship should not be influenced by any disturbance of kidney function nor by decomposition of diacetic acid within the urinary passages. However, it has been reported by Martin and Wick[4] that there is no definite relation between the concentrations of acetone in blood and urine with diabetic acidosis; they were inclined to doubt the accuracy of the methods used in early work on the excretion of acetone.

The recently devised method of Greenberg and Lester[5] is especially adapted to the study of this problem, since acetone is determined directly in the presence of diacetic acid. This method was employed in the present study. The volumes of reagents and samples were increased five-fold so that 10 ml of CCl_4 extract were obtained for reading in the Evelyn instrument. Digestions for total acetone were conducted under a condenser with ground glass connections.

Results are presented in the accompanying table of the work done on the admission specimens from a group of patients with diabetic ketosis; the first 5 cases were patients with diabetic coma.

Inspection reveals that the concentrations of acetone in blood and urine are consistently similar. These results, therefore, tend to support the view that acetone is excreted into the urine by the physical processes of filtration and diffusion. This view is also supported by the study of Lehman,[6] which has just appeared. He found similar concentrations of acetone in blood and urine following the intravenous administration of iso-propyl alcohol. Probably the failure of Martin and Wick to observe this relation was due to the fact that what they studied and reported as "acetone" was the fraction of the total acetone bodies precipitated by boiling with mercuric sulfate; this fraction represents acetone plus diacetic acid. It was pointed out by Widmark that the excretion of these 2 substances follows entirely different laws.

Two instances of diabetic coma with "renal block" are provided by Cases 3 and 4. In each case the total acetone body content of the urine was actually less than that of the blood; in each case the urinary test with ferric chloride was negative and that with nitroprusside only 2+. Since the concentrations of free acetone in blood and urine are similar, it would appear that either diacetic or oxybutyric acid is excessively reabsorbed by the renal tubules; the negative reaction with ferric chloride implicates diacetic acid. (The assumption is made that decomposition of diacetic acid in a slightly acid medium, during the flow through the urinary passages,

[4] Martin, Helen E., and Wick, A. M., *J. Clin. Inv.*, 1943, **22**, 235.

[5] Greenberg, L. A., and Lester, D., *J. Biol. Chem.*, 1944, **154**, 177.

[6] Lehman, A. J., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 232.

is not an important item).

These observations, therefore, indicate that the nature of the renal block is something of a functional disturbance due to an alteration of the tubule threshold for diacetic acid, as was suggested several years ago by Apple and Cooper.[7]

It is not at all clear why this phenomenon should occur in occasional cases. Often it is associated with oliguria, but oliguria is common in diabetic coma, and usually the concentration of diacetic acid in the urine is high. Dehydration and oliguria were more evident in Cases 1 and 2 than in these cases with negative ferric chloride.

[7] Apple, K. E., and Cooper, D. A., *Am. J. Med. Sc.*, 1927, **173**, 201.

Summary. A study has been made of the acetone bodies in blood and urine from a group of patients with diabetic ketosis, including 2 coma patients with urine giving a negative reaction with ferric chloride. The concentrations of free acetone in blood and urine were found to be similar in all cases; this observation supports the view that acetone is secreted into the urine by the physical processes of filtration and diffusion. The observed results were compatible with the suggestion that the cause of a negative urinary reaction with ferric chloride in certain cases of diabetic coma is due to a functional disturbance of the renal tubules, with elevation of the threshold for diacetic acid.

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Effect of Sulfathalidine and Sulfamethazine on Gaseous Distention in the Obstructed Small Intestine of Cats.

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It has been shown repeatedly that there are 3 sources of gas in intestinal distention (1) swallowed air, (2) decomposition of food, (3) diffusion, chiefly of nitrogen, from the blood into the intestines. According to Fine and his coworkers,[1] the nitrogen from the blood diffuses into the intestine in significant amounts only if other gases have collected in the gut in substantial volume. It has also been shown[1,2] that in the absence of food, swallowed air accounts for most of the gas present in the small intestine. The amount of swallowed air is variable within wide limits but can be controlled in great measure by withholding food and water and an inlying Levin tube. The fermentation or putrefaction of food is the second major source of gas in the obstructed small intestine. The coli-aerogenes-proteus group and certain clostridia, normally present in the small intestine, play a predominant role in this method of gas production. The inhibition of bacterial activity by antibacterial agents should contribute to a more complete control of meteorism. Hence a reduction in the number and metabolic activity of these groups of organisms might have a therapeutic effect.

Many of the sulfonamides now in use have a definite inhibiting action on enterobacteriaceae and clostridia, though each one to a different degree. Sulfathalidine (pthalylsulfathiazole) and sulfamethazine (dimethylsulfadiazine) clinically and experimentally have been shown to be effective against these

[1] Fine, J., and Levenson, W. S., *Am. J. Surg.*, New Series, 1933, **21**, 184.

[2] McIver, M. A., Benedict, E. B., and Cline, J. W., Jr., *Arch. Surg.*, 1926, **13**, 588.

bacteria.[3-6] The following experiments were performed to observe the effect of these drugs on the accumulation of gases in the obstructed small intestine of the cat.

Methods. Cats which had been deprived of food and water for 24 hours were subjected to laparotomy with the usual sterile precautions. Heavy cotton ligatures were used to completely occlude the oesophagus at the cardio-oesophageal junction to eliminate swallowed air and the ileum just proximal to the ileocaecal junction, thus producing a closed loop. Malted milk, which is a rich source of gas in the intestinal tract, was then injected into the stomach. Controls received 100 cc of malted milk without any drug; test animals received the same amount of malted milk containing sulfathalidine or sulfamethazine (1½ g per kg of body weight). At the end of 24 hours all animals were sacrificed and the amount of gas in the obstructed loop was measured by aspirating the entire contents into a syringe. The amount of gas in the stomach and in the small intestine was measured separately. Two animals were found dead at the end of 24 hours and were discarded. All the other animals were apparently in good condition at the time of execution.

A total of 46 animals were studied (Table I)—16 controls, 20 treated with sulfathalidine, and 10 with sulfamethazine.

Results. Gas production in the small intestine is apparently markedly depressed by the chemotherapeutic agents studied. Thus in the animals receiving sulfathalidine, the total gas production ranged from 0 to 60 cc, in those receiving sulfamethazine, from 0 to 30 cc, and in the control animals from 30 to 200 cc. Only 6 out of 16 controls showed less than 100 cc. These results seem quite significant, particularly considering individual variations in gas production.

TABLE I.
Total Gas in Closed Gastro-Intestinal Loops Containing Malted Milk.
A. Sulfathalidine; B. Sulfamethazine; C. No drug.

	A	B	C
	10	30	200
	0	15	75
	10	12	140
	0	0	110
	50	19	136
	0	0	100
	32	5	105
	16	7	200
	55	20	30
	8	12	55
	2		190
	60		130
	20		160
	0		57
	18		62
	3		39
	38		
	10		
	10		
	10		
Average	17.6	12.0	112.9

This observation is consistent with that of Sarnoff and Fine[7] who showed a protective effect by sulfasuxidine and sulfathalidine on isolated loops of ileum of dogs in which occlusion of the venous return produced gangrene except when these drugs were present in the injured loop.

Conclusions. Gas formation from malted milk in the occluded gastrointestinal tract is significantly depressed by sulfathalidine and sulfamethazine.

[3] Poth, Edgar J., and Ross, Charles A., *Texas Reports on Biol. and Med.*, 1943, **1**, 345.

[4] Poth, Edgar J., International Abst. Surg., 1944, **78**, 373.

[5] Schweinburg, F. B., and Yetwin, I. J., *New Eng. J. Med.*, 1944, **230**, 510.

[6] Schweinburg, F. B., and Yetwin, I. J., *J. Bact.*, 1945, **49**, 193.

[7] Sarnoff, Stanley J., and Fine, Jacob, *Annals Surg.*, 1945, **121**, 74.

15476

Reduction of General Activity in Male Albino Rats from Electro-Convulsive Shock.

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The use of electro-convulsive shock for the treatment of functional psychoses and certain other abnormal mental states is now widespread. Although its use appears to be well justified on clinical grounds, there is no wholly satisfactory explanation of the physiological changes through which the benefits are effected.[1] It seems probable, therefore, that many types of animal experiments will be needed to supplement human investigations in defining and, ultimately, explaining both the immediate and distant effects of electro-convulsive shock, at the psychobiological level.

Many investigators have noted a reduction in exploratory activity and rate of locomotion of rats in various learning situations, following the administration of electro-convulsive shock. Some of these reports are still unpublished;[2,3] others are summarized by Stainbrook in his recent review.[4] Consideration of these data led Stone[5] to conduct a small-scale study of the effects of electro-convulsive shock on the daily voluntary activity of 6 female rats, housed in the Slonaker-type of activity drum. He found consistent, temporary reductions of activity after shock. The present report is a continuation of this earlier study. Its main objective is to gather confirmatory data from which to make a more reliable estimate of the concurrent effects of electro-convulsive shock on the voluntary activity of male rats than was possible in the preliminary study on females.

Procedure. The convulsive shock was given by applying a 50 milliamperes, alternating current for 0.2 second through electrodes clipped on the ears of the rat. This consistently resulted in a grand mal seizure which started with a generalized tonic contraction of the entire body and then gradually passed into the clonic phase after about 10 seconds. The total period of the seizure proper was between 20 and 30 seconds. In the main, the typical syndrome and its most common variants were like those described in some detail by Golub and Morgan.[6]

The study began with 15 well-tamed males, approximately 70 days old. No shock was administered during the first 25 days of the study as it was desired that the animals become thoroughly adapted to their drums and the routine of handling before the introduction of shocks. From the first day and thereafter each animal was removed from its drum once daily and manipulated as much as would be necessary later on when shocks were to be administered. While the rat was out of the drum the experimenter replenished the supply of food and water, so that a surplus of these was available at all times. As a regular routine each rat was returned to the floor of the drum, ready for activity, whether or not he had been shocked.

Following the 25-day period, in which each rat tended to establish a characteristic level of daily activity, the first electro-convulsive shock was given. One seizure was induced daily in each rat on 5 consecutive days. This course was followed by a 5-day period in which no shock was given. Then followed (1) 2 additional 5-day periods of shock, alternating with 5-day periods of no shock, (2) one 5-day period of pseudo-shock, in which all manipulations except passage of the current occurred, and (3) a 20-day recovery period without

1 Kalinowsky, L. B., and Hoch, P. H., *Shock Treatments and Other Somatic Procedures in Psychiatry*, New York, Grune & Stratton, 1946.

2 Horowitz, M. W., and Stone, C. P., *J. Comp. Psychol.*, 1946, in press.

3 Stone, C. P., and Poplin, Betty, *J. Comp. Psychol.*, 1946, in press.

4 Stainbrook, E., *Psychol. Bull.*, 1946, **43**, 21.

5 Stone, C. P., Proc. Soc. Exp. Biol. and Med., 1946, **61**, 150.

6 Golub, L. M., and Morgan, C. T., *J. Comp. Psychol.*, 1945, **38**, 239.

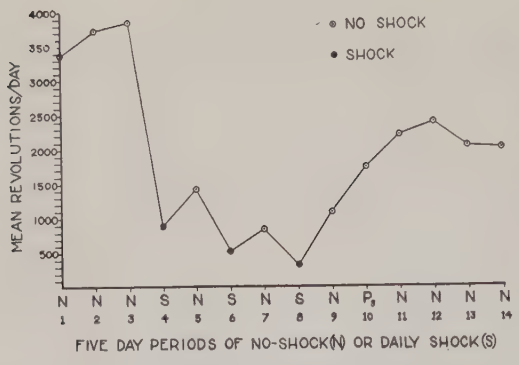

FIG. 1.
Activity of 13 male albino rats in revolving drums, based on the means for 5-day periods. In periods 4, 6, and 8 the rats received a daily electro-convulsive shock.

shock or pseudo-shock. Altogether there were 15 convulsive seizures, spread over a period of 25 days. Two of the rats were eliminated during the experiment. One died, we believe, from a disturbance of the respiratory mechanism. The other became paralyzed in the posterior third of the body, possibly, (but not certainly) from a spinal injury incurred at the time of shock.

Results. The records for the first 10 days of preliminary running were discarded because of the excessive variability of individuals while becoming adapted to the procedures. As shown in Fig. 1, 5-day periods have been numbered serially from 1 to 14. For each period the mean number of drum revolutions per rat per day has been calculated. The significances of differences between group means for the 5-day periods were calculated by use of the conventional small sample technic.

The mean level of activity during the first shock period (number 4) is significantly lower than that of the preshock periods 1, 2 and 3 ($P<0.01$). The same is true of shock periods 6 and 8. The mean of shock period 6 is significantly lower than the mean for no-shock period 5 ($P<0.01$); and the mean of shock period 8 is significantly lower than the means of no-shock periods 5 and 7. Thus, it is clear that the voluntary activity of the group is significantly reduced by 5 daily electro-convulsive shocks whether one considers the level of preshock activity or the levels reached in the intervening no-shock periods.

From period 4 to 8 there is a cumulative downward trend. Its consistency suggests that we have here a real cumulative effect, although the statistical significance of successive differences is less clear-cut than those referred to above (for the differences between periods 4 and 6, $P<0.05$; for periods 6 and 8, $P<0.10$; and for periods 4 and 8, $P<0.02$). In this experiment no rat was consistently hyperactive upon being returned to the drum after being shocked. Indeed, there was much less running on the part of all during the next 6 to 10 hours than had characterized their behavior at comparable times in the preshock period.

The period of pseudo-shock (number 10) is noteworthy because it is attended by a rise rather than a drop in activity. This would seem to indicate that the period was a constituent part of the course of recovery and that the convulsive shock was the essential cause of reduced activity during the periods of shock.

For the last 4 periods of no-shock the mean level of activity is fairly consistent, but lower than that of the preshock periods. Although a few rats were notably hyperactive, more of them were hypoactive, as compared with their preshock records. The net effect is a reduction of the combined mean value for periods 11, 12, 13 and 14 as compared with that of preshock periods 1, 2 and 3 ($P<0.05$). Possibly full recovery of some of the animals had not yet been achieved at the termination of our experiment, 30 days after the last shock. However, we are not warranted in drawing this conclusion without further appraisal of the natural reduction in activity of male rats during the 5th month of age. According to Richter,[7] the mean begins to decline between the 3rd and 4th month. If this should hold for our animals also, it is possible that a part or all of the drop in means for periods 11 to 14, as compared with periods 1 to 3, is due to the natural age decline in drum activity.

Finally, a word of caution should be urged against hasty assumption that specific factors accounting for reduction in drum activity, as

[7] Richter, C. P., *Comp. Psychol. Monog.*, 1922, **1**, pp. 55.

herein described, and the curtailment of exploration and increase of time scores as reported by others[4] are identical in kind. The literature is replete with examples showing that one and the same end-result in terms of reduction of level of activity may be the consequence of many diverse factors. Through factor analysis, van Steenberg[8] found that only a small fraction of the reliability of drum scores can be accounted for by a factor which is common to these and to other performances in mazes, problem boxes, and other tests. Thus, further analyses are needed before one may safely assume that the partial inactivation of rats in the activity drum is effected by the same factors as those which interfere with the exploratory tendency and the increase in time scores on learning tasks.

Summary. A series of daily electro-convulsive shocks in 13 male rats, for 5-day periods, significantly reduced concurrent activity as compared with (1) preshock activity, (2) 5-day periods of no-shock, interposed between periods of shock, and (3) the post-shock recovery period. There is a cumulative effect of successive periods of shock. The mean level of activity reached during the post-shock period of 30 days was significantly lower than that of the preshock period. Whether this difference was due to natural decline of activity with age or to lingering effects of electro-convulsive shock was not determined.

[8] van Steenberg, N. J. F., *Psychometrika*, 1939, **4**, 179.

15477 P

A Second Motor Nerve System to Frog Skeletal Muscle.

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The existence in skeletal muscle of special tonus mechanisms—particular nerve or muscle fibres or contractile elements—has often been proposed and as often been discarded. Tasaki and Mizutani[1] recently reported that stimulation of small diameter nerve fibres could initiate slow contractions in frog muscles. It appears that no electrical investigation of this problem has yet been made, nor have the structures giving slow contractions been identified. The present investigation following Tasaki and Mizutani's study clearly demonstrates a separate neuromuscular system which may well be involved in tonus and contracture and which has been missed because of the much greater action of the well-known twitch system. This latter has been eliminated (a) by progressive pressure block or galvanic block applied to the frog's sciatic below the point of stimulation following Leksell[2] (the large fibres failing sooner) and then observing various leg muscles, or (b) by cutting all but the desired fibre[1] in a small nerve (about 10 fibres) to an isolated toe muscle (extensor longus digiti IV., 15 mm long, 40 to 60 fibres) observed under the microscope.

As compared with the usual motor nerve fibres, those here active are of smaller diameter, higher threshold, greater resistance to pressure or polarization block and slower conduction. They are probably not autonomic, since they may be larger than 5 micra. A single impulse in one evokes a barely visible movement of an attached small muscle, repeated impulses at 3 to 4 a second give a small local shortening and at 20 to 50 a strong slow local contraction appears and in some preparations an occasional fully-propagated

* Seymour Coman Fellow. The present investigation was aided by grants from the Seymour Coman Fund and the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

[1] Tasaki, I., and Mizutani, K., *Jap. J. Med. Sciences*, 1944, **10**, 237.

[2] Leksell, L., *Acta Physiol. Scand.*, 1945, **10**, suppl. 31, 84.

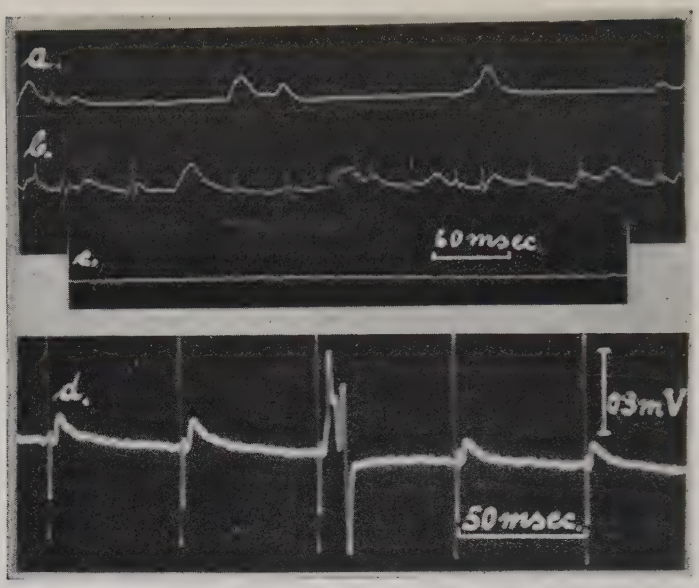

FIG. 1.

a. M. semitendinosus. Spontaneous activity recorded during "rest" in circulated spinal preparation. *b.* Same preparation; activity following on weak reflex excitation (touching of toes). Note increased frequency of local potentials and appearance of some propagated impulses. The latter are accompanied by small twitches. *c.* Activity abolished after cutting nerve supply. *d.* M. extens. long. dig. IV. Potentials set up by stimulation of a single small nerve fiber at 20 per sec. Occasional propagated impulses appeared during this series. Amplification the same in all records.

twitch may also be set up (Fig. 1d). Along with the accompanying potentials the local shortening is limited to the region of the neuromuscular junction.

The local muscle potential resembles the usual endplate potential as obtained after curarization.[3] It appears after a longer interval following stimulation of the nerve, is often slower on rise and fall, and is smaller than the curarized endplate potential. The local potential rises, to a peak of approximately 0.2 mV when excessive short-circuiting is avoided, in 3 to 4 msec. and falls to half in about 13 msec. When recording the activity from the surface of exposed "resting" muscle, local potentials of widely varying time course appear (Fig. 1a). These do not represent the true time course of individual potentials but are a composite effect of multiple scattered units. Repetitive stimulation at 20 to 50 per sec. does not lead to significant local potential facilitation. A similar system of local contractions and potentials has recently been studied in crustacea.[4]

Although the local and propagated muscle responses are so different, the same muscle fibres may be involved in both. The occasional appearance of fully conducted twitch responses with the local potentials on tetanizing of a small nerve fibre has been mentioned (Fig. 1d). Further, dissection of small muscle bundles shows that the muscle fibres are all similar and that the same ones can be excited by "small" and "large" nerve fibres. Both types of nerve endings, therefore, can occur on one muscle fibre. One small nerve fibre can supply multiple groups of endings, each group reaching several muscle fibres in a single area, as revealed by as many as three regions of local potential and shortening.

The local potentials, with or without propagated ones, can easily be obtained from the surface of many leg muscles in the circulated spinal frog. They appear at varying fre-

[3] Eccles, J. C., Katz, B., and Kuffler, S. W., *J. Neurophysiol.*, 1941, **5**, 362.

[4] Katz, B., and Kuffler, S. W., *Proc. Roy. Soc.*, 1946, B 133, 374.

quencies (Fig. 1a,b) depending on the number of sensory impulses reaching the cord from the periphery. They can develop tensions of 10 to 15% of a maximal twitch response. Nerve section abolishes them (Fig. 1c). Mammals have not yet been studied.

Summary. A separate small-nerve motor system to frog skeletal muscles is described. It is active reflexly and may be related to muscle tone. It evokes local potentials and local shortening at the region around the nerve-muscle junctions, in sharp contrast to the propagated twitches and muscle impulses which can be elicited, by the usual motor nerves in the same muscle fibres. Small fibre activity alone may set up appreciable muscle tension.

I wish to thank Dr. R. W. Gerard for his stimulating interest and help and also Mr. L. Boyarsky for much assistance.

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Benadryl* Fails to Protect Against the Histamine-Provoked Ulcer.†

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It has been shown that synthetic benzhydryl alkamine ethers are capable of preventing fatal asthma induced in guinea pigs by administration of histamine either intravenously or by inhalation of atomized aqueous solution.[1] These drugs also have been demonstrated to be effective in alleviating anaphylactic shock in guinea pigs[2] and dogs.[3] Lowe and his associates also found that the activity of the synthetic benzhydryl alkamine ethers equaled or exceeded that of the 2 Fourneau histamine antagonists, thymoxyethyldiethyl amine (929F) and N-phenyl-N-ethyl-N′-diethylenediamine (1571F).[2] The latter drugs (929F) and (1571F) have been shown to produce no alteration of gastric response to histamine in Heidenhain pouches in dogs.[4,5]

Of the various synthetic benzhydryl alkamine ethers, β-dimethylaminoethyl benzhydryl ether hydrochloride (benadryl) was found to be the most potent histamine antagonist in alleviating anaphylactic shock and histamine-induced asthma in guinea pigs.

The effect of benadryl on the action of histamine as judged by blood pressure changes has been studied.[6] It was found that benadryl given intravenously in the amounts of 3 mg per kg body weight abolishes the fall in blood pressure in dogs produced by 0.001 to 0.002 mg per kg body weight of histamine. In that study, the amount of histamine "antagonized" by given doses of benadryl was quantitated and the mechanism of this "antagonism" was stated to be owing to the adsorption of benadryl onto the site of action of histamine, a circumstance which Wells and his associates believes disturbs the histamine equilibrium such that a given amount of his-

* Benadryl was supplied through the courtesy of Parke-Davis Company.

† The researches upon which this presentation is based were supported by the Augustus L. Searle Fund for Experimental Surgical Research, the Citizens' Aid Society, the Robert A. Cooper Fund for Surgical Research, and by grants of the Graduate School of the University of Minnesota.

1 Loew, E. R., Kaiser, M. E., and Moore, U., *J. Pharm. and Exp. Therap.*, 1945, **83**, 120.

2 Loew, E. R., and Kaiser, M. E., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 235.

3 Wells, J. A., Morris, H. C., and Dragstedt, C. A., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 104.

4 Burchell, H. D., and Varco, R. L., *J. Pharm. and Exp. Therap.*, 1942, **75**, 1.

5 Hallenbeck, C. A., *Am. J. Physiol.*, 1943, **139**, 329.

6 Wells, J. A., Morris, H. C., Bull, H. B., and Dragstedt, C. A., *J. Pharm. and Exp. Therap.*, 1945, **85**, 119.

tamine has much less opportunity to reach and combine with its site of action.

The effect of benadryl on gastric secretion stimulated by histamine in dogs with Heidenhain pouches has been reported to show a reduction of approximately 40% in gastric secretion in 3 of the 4 dogs studied (8 out of 12 experiments).[7]

It is the purpose of this study to determine whether benadryl is effective in altering the gastric secretory response to histamine, or in protecting against the histamine-provoked ulcer in dogs.

Methods and Experiments. Seventeen dogs were used in 2 series of experiments.

Series I: In the first series 2 Pavlov (innervated) and 2 Heidenhain (denervated) pouch dogs weighing 15 to 30 kg were used. The animals were fasted 18 hours previous to each experiment. Samples of gastric secretion were obtained every 30 minutes for 3 hours after histamine stimulation and studied for volume, free hydrochloric acid, and total acid.

Six sets of observations were made:

(1) Study of the gastric response to 0.5 mg aqueous histamine administered subcutaneously for standardization of the experiments. (12 experiments).

(2) Study of the gastric response to the same dose of histamine subcutaneously, the animals having received benadryl orally in doses of 5 to 10 mg per kg body weight 30 minutes previously. (12 experiments).

(3) Study of the gastric response to the same dose of histamine subcutaneously, the animals having received benadryl subcutaneously in aqueous solution in doses of 10 to 30 mg per kg body weight 20 minutes previously. (24 experiments).

(4) Study of the gastric response to benadryl alone in doses of 5 to 10 mg per kg body weight administered orally and subcutaneously. (8 experiments).

(5) Study of gastric response to histamine-in-beeswax mixture alone (30 mg base) as prepared after the method of Code and Varco[8] administered intramuscularly. (4 experiments). In this way, gastric response over a 24-hour period was studied.

(6) Study of the gastric response to the simultaneous injections of histamine-in-beeswax (30 mg base intramuscularly) and benadryl-in-beeswax mixture (100 mg intramuscularly) prepared in the same manner. (4 experiments).

Series II: Thirteen healthy intact dogs weighing 10 to 25 kg were used.

Seven dogs were given daily intramuscular injections of histamine-in-beeswax (30 mg base) and benadryl-in-beeswax (100 mg) simultaneously several hours after feeding. As controls, 4 dogs were given daily intramuscular injections of histamine-in-beeswax alone (30 mg) and 2 dogs were given daily intramuscular injections of benadryl-in-beeswax alone (100 mg). Animals surviving 40 days were sacrificed at that time; others died or were sacrificed when signs of impending death were present.

Results. Series I: The results of the studies on gastric secretion can be summarized by stating that in 40 experiments benadryl had no demonstrable effect on the secretory response to histamine. There was no evidence of decrease in volume or acidity of the secretion with benadryl premedication. Some of the data indicated that there was a slight increase in quantity and acidity with slight prolongation of the gastric secretion in response to histamine after benadryl administration.

Series II: (Table I). Results of simultaneous daily injections of histamine-in-beeswax and benadryl-in-beeswax show that all (7) dogs developed gastric and/or duodenal ulceration. The 4 dogs receiving histamine-in-beeswax alone developed ulceration. Of the 2 dogs receiving benadryl-in-beeswax alone, one died after 19 daily injections demonstrating early gastric erosions and petechial bleeding points; the other, sacrificed at 40 days, showed no demonstrable pathology.

The 7 animals receiving both drugs were noted to become more debilitated as evidenced by greater weight loss, anorexia and listlessness than those receiving histamine alone. This occurrence also is reflected in

[7] Loew, E. R., MacMillan, R., and Kaiser, M. E., *J. Pharm. and Exp. Therap.*, 1946, **86**, 229.

[8] Code, C. F., and Varco, R. L., *Am. J. Physiol.*, 1942, **137**, 225.

TABLE I.
Results of Daily Injections of a Mixture of Benadryl-in-beeswax and Histamine-in-beeswax.

Dog No.	Wt in kg	Daily dose of benadryl, mg	Daily dose of histamine base, mg	No. of inj.	Results
1	25	100	30	14	Duodenal erosions
2	20	100	30	5	Three duodenal ulcers, one perforated
3	19	100	30	40	Duodenal ulcer
4	16	100	30	40	" "
5	13	100	30	40	" "
6	10	100	30	29	" " Hemorrhagic gastritis
7	13	100	30	40	Duodenal ulcers
Controls					
8	24	0	30	40	Gastric ulcer Duodenal ulcer
9	23	0	30	40	Gastric ulcer Duodenal ulcer
10	25	0	30	40	" "
11	13	0	30	40	" "
12	20	100	0	19	Gastric erosions Petechial bleeding points
13	15	100	0	40	Negative

the circumstance that only 4 of the 7 dogs survived 40 days, while all 4 receiving only histamine survived 40 days. The 2 receiving benadryl alone survived 19 and 40 days. One dog receiving both drugs died after 5 daily injections of perforated duodenal ulcers with peritonitis. One was sacrificed because of impending death after 14 daily injections of both drugs and showed duodenal erosions, and one sacrificed after 29 daily injections showed duodenal ulcers and hemorrhagic gastritis.

Discussion. Evidence of untoward reactions to benadryl were observed in occasional instances consisting of vomiting when given orally in doses of 10 or more mg per kg of body weight; therefore, we were unable to evaluate the effect of larger doses by this route.

Administration by the subcutaneous route was used to study the effect of larger dosages of benadryl. These dosages were as great and greater than those calculated to be capable of "antagonizing" 0.5 mg of histamine as judged by blood pressure response.

The fact that some of the data (approximately 40%) showed increased volume and acidity where benadryl premedication was given over the response to histamine stimulation alone is not of great significance because the increase was not great. In this respect, benadryl, when given alone, produced neither an increase nor a decrease in the level of gastric secretion in fasting pouch dogs.

The results found in this study, together with the failure of a consistent decrease in gastric secretion as reported by others[7] indicate that benadryl is not a specific antagonist of histamine, but counteracts *some* of the effects of histamine by virtue of its own pharmacological action.

Conclusions. 1. Benadryl fails to alter the gastric secretory response to histamine stimulation in pouch dogs. 2. Benadryl (given in 100 mg doses in beeswax mixture intramuscularly) fails to protect against the histamine-provoked ulcer in dogs.

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Cultivation of *Bacterium tularense* in Peptone Media.*

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(Introduced by K. H. Lewis.)
From Camp Detrick, Md.

Until recently *Bacterium tularense* has resisted attempts at cultivation in any fluid medium or on any but complex solid media[1] and until recently no dilution plate counting method was described for this organism. Since 1942 there have been described several semi-synthetic liquid media,[2,3] and a more easily prepared fluid medium containing heart infusion broth, hemoglobin, glucose and cystine.[4] Larson[5] has further modified this last medium by replacing the hemoglobin with a completely soluble erythrocyte extract, and has quantitatively confirmed earlier suggestions[2,4] that large inocula are required for propagation in liquid media.

Through a process of elimination and substitution, starting from Rhamy's hemoglobin cystine heart agar[6] we found it possible to derive more simple fluid and solid media quite satisfactory for routine use. These consist of 2% Difco Bacto Peptone, 1% sodium chloride and 0.1% glucose, with or without 2% Difco Bacto Agar. The hydrogen-ion concentration is not adjusted before sterilization. The only precaution that need be carefully observed is the use of relatively large inocula.

The 28 strains which were investigated are listed in Table I in order of decreasing virulence. All strains were originally isolated from human sources and presumably were highly virulent at the time of isolation. Most of the strains, together with information concerning their histories, were supplied us by Professor Lee Foshay. The exceptions are Strain Ince (Professor Cora M. Downs) and Strain 38 (National Institute of Health). The donors' designations are used throughout. Virulence titrations were carried out by injecting groups of 6 mice intraperitoneally with appropriate 10-fold serial dilutions of suspensions of equivalent turbidity (T-500 Fullers earth standard, National Institute of Health). The 50% lethal doses were calculated by the method of Reed and Muench[7] from the percentage mortality within 10 to 14 days, and are recorded as dilutions of the standard suspension. We are indebted to Capt. L. L. Coriell for many of these titres, which are taken from his unpublished data.

Peptone broth was tested for its ability to permit the growth of the 28 strains, using 50 or 100 ml of medium in 250 ml Erlenmeyer flasks. The primary cultures were inoculated into this medium sufficiently heavily from stock blood cysteine agar slants to produce a faint turbidity, and the remaining transfers were made with sterile pipettes. All strains were tested for at least 9 consecutive transfers before terminating the experiment. Twenty-one strains could be maintained by the use of 1% inocula (*i.e.*, one ml of 24-hour broth culture as inoculum for 100 ml sterile broth). A few representative strains of this group (Dieck, Ince and Camp) have been carried through more than 20 transfers, and Strain Schu through 98 transfers, without difficulty. The remaining 7 strains required a larger inoculum for serial cultivation, 10% being successful in all cases. Strain 38, representing this group has been maintained through about 20 transfers under these conditions. With one exception (Strain LR),

* Studies conducted at Camp Detrick, Frederick, Md., between February, 1944, and June, 1945.

1 Francis, E., *J. Bact.*, 1942, **43**, 434.

2 Berkman, Sam, *J. Infect. Dis.*, 1942, **71**, 201.

3 Tamura, J. T., and Gibby, I. W., *J. Bact.*, 1943, **45**, 361.

4 Steinhaus, E. A., Parker, R. R., and McKee, M. T., *U. S. Pub. Health Rep.*, 1944, **59**, 78.

5 Larson, C. L., *U. S. Pub. Health Rep.*, 1945, **60**, 863.

6 Rhamy, *Am. J. Clin. Path.*, 1933, **3**, 121 (cited by *Manual of Dehydrated Culture Media and Reagents*, 7th Edition, Difco Laboratories, Inc., Detroit, Mich., 1943).

7 Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

TABLE I.
Characteristics of Strains Investigated.

Strain	Area and year of isolation	50% lethal dose (ml standard suspension)	Growth with 1% transfers
Chr*	Ohio, 1937	<10-10	+
Gib*	Ohio, 1941	<10-10	+
Ri*	Virginia, 1932	<10-10	+
Holt*	Kentucky, 1944	10-10.6	+
Fox	Ohio, 1940	10-9.8	+
Dieck*	U. S. A., 1944	10-9.6	+
Camp*	Kentucky, 1944	10-9.5	+
Carr*	U. S. A., 1944	10-9.5	+
Ince*	Kansas, 1944	10-9.5	+
Schu*	Ohio, 1941	10-9.5	+
Scherm*	U. S. A., 1944	10-9.2	+
Coll*	U. S. A., 1945	10-8.8	+
Chur	Ohio, 1941	10-8.6	+
LR	Arkansas, 1927	10-8.5	—
Hugh	Ohio, 1940	10-6.3	+
Jap	Japan, 1926	10-4.4	—
HD	Austria, 1935	10-4.0	+
Sto*	Ohio, 1937	10-3.0	+
PF*	Ohio, 1936	10-2.5	+
Russ	Russia, 1928	10-2.0	+
De P	Ohio, 1938	>10-2.0	+
Die	California, 1938	>10-2.0	—
Max	Russia, 1928	10-1.7	—
Pi	Ohio, 1938	10-1.0	+
Ohara	Japan, 1931	10-1.0	—
26	Utah, 1921	10-1.0	—
Li*	Canada, 1934	>10-1.0	+
38	Utah, 1920	avirulent	—

* Yield in peptone broth determined by dilution plate count.

strains requiring an inoculum in excess of 1% were found among those of lesser virulence.

All strains appeared to grow quite as luxuriantly in the 1st and 2nd passages in peptone broth as in later ones, indicating that no process of adaptation was required. The yields of 14 strains (indicated in Table I by asterisks) could be determined by means of a dilution plate counting method described elsewhere.[8] All produced one to 5 billion viable organisms per ml in 24 to 48 hours at 37°C in 50 ml cultures. The effect of aeration on yield was demonstrated with Strains Schu, Camp, Dieck and Ince. An increase in the volume of culture from 50 to 100 ml in 250 ml Erlenmeyer flasks decreased the yield to between 200 millions per ml and 800 millions per ml, while active aeration by means of spargers or by constant shaking increased it to between 3 and 10 billions per ml in 24 hours. Incubation at 23 to 25°C decreased the growth rate, but similar or slightly higher final yields were obtained after 48 to 72 hours. The remaining 14 strains produced roughly equivalent turbidity, but the yields could not be determined by the plate-counting method.

The effect of alterations in the composition of the fluid medium was investigated, using Strain Schu. The average yield was halved by decreasing the peptone concentration to 1%, but some growth occurred with as little as 0.25%. Bacto Peptone could be replaced by Proteose Peptone, Proteose Peptone No. 3 or Bacto Tryptose, but not by Neopeptone or Bacto Tryptone. No difference in yield was observed with 10 different lots of Bacto Peptone. Two and 4% corn steep liquor supported somewhat less luxuriant growth than 2% peptone. Sodium chloride was found to be approximately optimum over the range 0.4 to 2.0% in the presence of one and 2% peptone, but 0.2% gave a poor yield. Glucose was included because there is evidence that

[8] Snyder, T. L., Engley, F. B., Jr., Penfield, R. A., and Creasy, J. C., *J. Bact.*, 1946, **52**, 241.

TABLE II.
Effect of Inoculum Size and Reducing Agents on Growth of Strain Schu in Peptone Broth.

Reducing agent	Inoculum (No. of cells per 100 ml flask)	No. of flasks inoculated	No. of flasks turbid after incubation (hrs)						
			16	24	40	48	88	96	336
0	380,000,000	2	2	2	2	2	2	2	2
	38,000,000	3	3	3	3	3	3	3	3
	3,800,000	3	0	0	0	0	2	3	3
	380,000	2	0	0	0	0	0	0	0
0.1% cysteine-HCl	38	3	0	0	3	3	3	3	3
	3.8	3	0	0	3	3	3	3	3
	0.38	3	0	0	2	2	2	2	2
	0.038	3	0	0	0	0	0	0	0
0.01% thioglycollate	38	3	0	0	3	3	3	3	3
	3.8	3	0	0	0	3	3	3	3
	0.38	2	0	0	0	0	0	0	0
	0.038	3	0	0	0	0	0	0	0

it is utilized,[1,9] but our results did not indicate any stimulation by either 0.1 or 1.0%. Variation of the hydrogen-ion concentration over the range pH 6.5 to 7.2 appeared to have no effect.

Only Strains 38, Schu and Chur were tested on peptone agar slants. These strains were twice carried through 20 successive passages without difficulty before terminating the experiment. Transfers were usually made at 24-hour intervals by spreading a 2 mm loopful of growth over the surface of the sterile slant. Growth appeared to originate within streaks of the inoculum, thence spreading over the entire surface. The yield appeared equivalent to that on Rhamy's medium,[6] but was somewhat less than that obtained with blood cystine agar.[10]

The effect of continued cultivation on retention of virulence was investigated only with Strains Schu and Chur. Titrations were carried out as described above, but in this case plate counts made on the same suspensions permit us to report the 50% lethal doses in terms of numbers of organisms. Repeated titration of stock cultures of these strains gave endpoints falling within the range 0.2 to 3.0 organisms. No decrease in virulence was detectable with either strain after 34 passages on peptone agar at 37°C, or with Strain Chur after 30 passages in peptone broth at 37°C. Broth cultures of Strain Schu were studied more extensively. Of 5 separate series, each carried through 50 passages at 25°C, none showed any decrease in virulence, whereas of 5 series carried through 50 passages at 37°C, 3 showed no loss and 2 had decreased to such an extent that the 50% lethal doses were 130 thousand and 11 million organisms respectively.

The minimum effective inoculum for consecutive cultivation in peptone broth was determined more precisely in the case of Strains 38 and Schu, which were selected as representatives of the 2 groups previously described. In this study, the incubation periods were held constant at 24 hours and the inocula varied from 0.1 to 10.0%. Strain 38 could be carried only through one or 2 transfers with 5% or smaller inocula, whereas the control series was maintained through 20 passages with 10% inocula before being discarded. Strain Schu could be maintained indefinitely with 1% inocula, but the 0.3% inoculum series failed to become turbid within 24 hours in the 3rd passage, and the 0.1% series in the second. On the basis of plate counts of 24-hour cultures it was possible to estimate that the minimum inoculum for Strain Schu under these conditions was about one to 4 million organisms per ml of fresh medium.

The constancy of the minimum inoculum size as a characteristic of the strain is open to question. Strain 38 was re-examined after

[9] Downs, C. M., and Bond, G. C., *J. Bact.*, 1935, **30**, 485.

[10] Francis, E., *J. Am. Med. Assn.*, 1928, **91**, 1155.

2 years cultivation on blood cysteine agar and still required an inoculum in excess of 1%, but one series of Strain Schu has shown a decrease in the minimum inoculum size to less than 30 organisms per ml after 43 or less passages in peptone broth. This cannot be referred to the effect of a different lot of medium since a control series in the same lot demonstrated the usual requirement for a large inoculum.

Three independent lines of investigation appear to relate the dependence of *Bacterium tularense* on large inocula to an inhibitory action by oxygen or by elevated oxidation-reduction potential of the medium. This is indicated by the effect of reducing agents and decreased oxygen tension on the size of inoculum required, and by the form of growth which occurs in agar shake cultures.

Table II shows the effect of reducing agents. Both cysteine and thioglycollate decreased the minimum effective inoculum of Strain Schu from between 4,000 and 40,000 organisms per ml to approximately one organism per flask. These compounds have also been shown to permit the growth of "strict" anaerobes in fluid cultures exposed to air,[11,12] and to decrease the minimum effective inoculum of certain facultative species.[13]

The effect of oxygen tension was investigated by inoculating peptone agar slants with serial 10-fold dilutions of Strains 38 and Schu and incubating 5 days in mixtures of air and nitrogen. Undiluted commercial nitrogen (assumed to contain 0.5% oxygen) reduced the minimum effective inoculum of Strain 38 to approximately 40,000 per slant, as compared with 4,000,000 per slant in air (21% oxygen). Strain Schu was less affected, the minimum inoculum being reduced from 400 per slant to about 4 per slant, with the optimum at an estimated 1.25% oxygen. These oxygen tensions are within the range which permits the growth of several "obligatory" anaerobes on ordinary media.[14]

In peptone agar shake cultures (0.5 to 1.0% agar) growth of *Bacterium tularense* tended to occur in a narrow zone parallel to, but separated from the surface. This was a constant observation with Strain 38, in which case the zone was always at least 10 mm below the surface and increased in depth as the size of the inoculum was decreased. The zone was about one mm thick after 24 to 48 hours, but increased by extension toward the surface and was several mm thick after a week. Strain Schu demonstrated similar growth stratification with dilute inocula (5000 organisms per ml or less), but larger inocula produced zones which approached the surface as the inoculum increased, until 500,000 to 5,000,000 organisms per ml showed only a one mm thick zone contiguous with the surface. The presence of cysteine caused the growth zone to originate nearer the surface with all inocula, and to extend thereto within 72 hours.

An apparently identical growth formation in agar shake cultures has been described for *Brucella abortus*, and has been ascribed to this species' requirement for carbon dioxide.[15] The preceding experiments on the effect of reducing agents and air-nitrogen mixtures on *Bacterium tularense* cannot lead to a similar conclusion, nor were we able to demonstrate any stimulation by the 2 or 3% carbon dioxide atmosphere obtained by burning a candle in a closed jar. Similar stratification of growth has been observed with a few other bacterial species in ordinary media (see Braun[16] and Prévot[17]) and ascribed to oxidation-reduction potential requirements,[18] and can be induced with numerous facultative anaerobes by the

11 Quastel, J. H., and Stephenson, M., *Biochem. J.*, 1926, **20**, 1125.

12 Valley, George, *J. Bact.*, 1929, **17**, 12.

13 Dubos, René, *J. Exp. Med.*, 1929, **49**, 559; 1930, **52**, 331.

14 McLeod, J. W., *Acta path. microbiol. Scand.*, 1930, **20** (Suppl. III), 255.

15 Wilson, G. S., *British J. Exp. Path.*, 1931, **12**, 152.

16 Braun, H., *Schweiz. Z. allgem. Path. Bakt.*, 1938, **1**, 201, 257, 267; 1939, **2**, 309.

17 Prévot, A. R., *Ann. Sci. Natur.*, Ser. Bot., 1933, **15**, 23.

18 Prévot, A. R., *C. R. Soc. biol.*, 1938, **127**, 489.

use of heavy-metal precipitants,[16,19,20] high oxygen pressures,[21] or decreased concentration of nutrients.[22]

The preceding observations appear to relegate *Bacterium tularense* to the poorly defined group of "microaerophiles," as was suggested at one time by Foshay.[23] The species is apparently unusually sensitive to mildly oxidizing intensity in the environment. Our data do not indicate whether this is referable directly to molecular oxygen, or indirectly through the reaction of oxygen with constituents of the medium. As in the case of obligatory anaerobes and of other "microaerophiles," inhibition tends to be abolished by reducing agents, decreased oxygen tension or large inocula. On the other hand, the need for oxygen is indicated by the absence of growth below characteristic levels in agar shake cultures, and stimulation of growth by oxygen is shown by the increased yields obtained in aerated broth cultures.

Summary. 1. *Bacterium tularense* grew luxuriantly and retained virulence during consecutive transfers in peptone broth and on peptone agar without blood or blood constituents. 2. Large inocula were required with the strains tested. The minimum effective inoculum was decreased by reducing agents or by a lowered oxygen tension over the culture, but growth was found to be dependent upon oxygen and stimulated by increased aeration. 3. *Bacterium tularense* appears to be an obligatory aerobe sensitive to mildly oxidizing environment.

[19] Burnet, F. M., *J. Path. Bact.*, 1927, **30**, 21.

[20] King, J. W., and Rettger, L. F., *J. Bact.*, 1942, **44**, 301.

[21] Williams, J. W., *Growth*, 1939, **3**, 21.

[22] Cahn-Bronner, C. E., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 454.

[23] Foshay, Lee, *Am. J. Clin. Path.*, 1933, **3**, 379.

15480

Exudative Trypanosome Pleuritis of Mice Infected Experimentally with *Trypanosoma cruzi.*

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In the course of the routine transfer of a strain of *Trypanosoma cruzi* from mouse to mouse it was found that some of the animals developed a pleuritis which seemed to be of specific origin. Since we were unable to find a description of this pathological condition in the literature, our observations are presented in this note.

The strain of *T. cruzi** was carried in mice since August 1942 and was regularly transferred by subcutaneous, occasionally by intraabdominal, injection of blood containing the parasites. The virulence of the strain at the time when the occurrence of pleuritis was first noted had been increased by frequent passages through mice of 3 weeks of age (9-13 g). All the mice injected subcutaneously with 0.05 cc blood containing approximately 50 trypanosomes per microscope field (about 300 × lin. magnification) died within 10-17 days. In general, trypanosomes appeared in the blood of infected animals 2-8 days after inoculation, according to the number of organisms present in the inoculum.

The first observation was made in a young mouse (No. 234) which was inoculated subcutaneously the 28th of May, 1945 and died the 16th of June. At the autopsy ca. 0.5 cc of a clear pleural exudate was found containing 20-30 trypanosomes per microscope field; 0.1 cc of this exudate was injected intraperitoneally into another mouse which showed prostration 13 days later. Its blood contained at this period a very large number of trypanosomes. The animal was sacrificed and

* Strain of the *T. cruzi* (Chagas 1909) was received from the American Type Culture Collection.

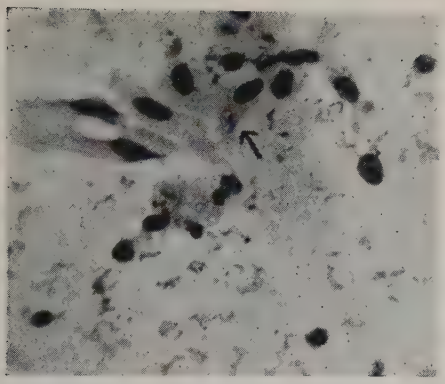

Fig. 1.
Pleural exudate of mouse No. 275, 11 days after subcutaneous infection with *T. cruzi*. Free trypanosome forms. Intracellular leishmania-like bodies in endothelial cells. (←)

the presence of a pleural exudate containing a large number of trypanosomes was noted. No bacteria could be cultured from this exudate on blood agar plates.

A similar observation was made later on in another mouse; No. 247. This animal was infected subcutaneously on the 5th of January, 1946 with 2 drops of blood containing 9 trypanosomes per microscope field. The number of parasites increased progressively in the blood: 6 days after inoculation: 2 trypanosomes per 100 fields; 17 days after inoculation: 4 parasites per one field. The mouse died on the 17th day showing a pleural exudate containing 1-3 trypanosomes per microscope field and a few leishmania-like forms. One drop of the exudate injected subcutaneously to 2 mice lead to a lethal infection. Microscopic sections of the heart and spleen of mouse No. 247 revealed the presence of numerous leishmania-like bodies.

The microphotograph (Fig. 1) of the pleural exudate of a young mouse shows the presence of free trypanosome forms and intracellular leishmania-like bodies. The animal (mouse No. 275; 12 g) died 11 days after subcutaneous infection with an inoculum of 0.05 cc of a suspension containing 50 parasites per field. The number of trypanosomes in the peripheral blood increased from about 14/field on the 3rd day to 160/field shortly before the death. Autopsy revealed the presence of 0.1 cc pleural exudate. Smears of the spleen showed numerous leishmania-like forms. Transfers of the exudate on blood agar did not show growth of bacteria.

In order to obtain information on the incidence of pleuritis in mice infected with *T. cruzi,* 26 young and 9 adult mice were infected subcutaneously. They all showed trypanosomes (2-22 per 100 fields) within 3 days, when the first examination was made. From this group of 35 mice, 25 died 9-17 days after infection, in 14 of them pleural exudate containing a large number of trypanosomes was noted. The volume of the exudate varied between 0.1 and 0.5 cc. Eight of the remaining 10 mice were sacrificed 9-11 days after the infection; in 3 of them pleural exudate with numerous trypanosomes was found. Thus, altogether 51.5% of the 33 mice examined presented pleural exudate containing trypanosomes and leishmania-like forms. In the adult group, 3 out of 9 mice developed pleuritis.

A survey recently conducted by G. Soo-Hoo in this laboratory showed that 18 out of 46 young mice (12-14 g) developed pleuritis (39%) after a heavy infection with a suspension containing approximately 50 parasites per microscope field. Out of another group of 56 mice which died after having received a light infection (5-10 parasites per field) only 2 animals (3.6%) showed exudate in the pleural cavity.

Summary. In the course of the infection of young mice with *T. cruzi* the incidence of pleuritis was observed in 3.6 to 50% of the cases depending on the size of the inoculum. The serous exudate which was sterile if examined with bacteriological methods contained numerous trypanosome forms and intracellular leishmania-like bodies.

15481 P

Further Evidence for Methylatable Precursors of Choline in Natural Materials.*

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In the course of work on choline metabolism in chicks the choline content of several diets was determined by both a colorimetric method and a microbiological method. The colorimetric method was essentially that of Thornton and Browne[1] using reineckate precipitation. Ethanol was used to extract the choline-bearing substances from the original materials. Hydrolysis was carried out with 3% H_2SO_4 and the precipitation was done after neutralization to pH 6.0 with solid $Ba(OH)_2$ and then to pH 7.5 with 1 N NaOH. The microbiological method was that of Horowitz and Beadle[2] using the Neurospora "cholineless" mutant 34486. The standards as well as unknowns were passed through the adsorption and elution steps. Reproducibility was poor with the Neurospora method on some samples. The results are shown in Table I.

On the purified and semipurified diets the 2 methods were in close agreement. On the practical diet with liver meal and the simplified diet, however, the Neurospora method gave substantially higher values. The simplified diet contained 15% peanut meal.

The value of >0.08% obtained for the simplified diet by the Neurospora method is in essential agreement with that published by McGinnis, Norris and Heuser.[3] The value of <0.04% obtained by the reineckate method is in agreement with a value calculated from the choline contents of the individual ingredients as published by Engel.[5]

The higher value obtained by Neurospora on the practical diet containing liver meal might be expected in view of the results of Jukes and Oleson.[6] These authors found that, if a crude aqueous extract of liver was hydrolized with $Ba(OH)_2$ and then precipitated with reineckate, the resulting filtrate was active for Neurospora.

Jukes and Dornbush[7] observed that dimethylaminoethanol stimulated the mutant Neurospora in a manner similar to choline. Apparently, however, dimethylaminoethanol was not precipitated by reineckate when pure and was only partially precipitated in the presence of choline. These results suggest that those diets on which the higher values were obtained by the Neurospora method in the present study contain dimethylaminoethanol or related compounds. Jukes and Oleson[8] have called attention to the fact that dimethylaminoethanol has been isolated from one member of the Leguminosae.

Numerous compounds were tested with 2 Neurospora "cholineless" mutants by Horowitz, Bonner and Houlahan.[9]. Their data

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† Now with the Institute of Statistics, University of North Carolina, Raleigh, N.C.

1 Thornton, M. H., and Browne, F. K., *Ind. Eng. Chem., Anal. Ed.*, 1942, **14**, 39.

2 Horowitz, N. H., and Beadle, C. W., *J. Biol. Chem.*, 1943, **150**, 325.

3 McGinnis, J., Norris, L. C., and Heuser, G. F., Proc. Soc. Exp. Biol. and Med., 1944, **56**, 197.

4 Lucas, H. L., Norris, L. C., and Heuser, G. F., *Poultry Science*, 1946, **25**, 373.

5 Engel, R. W., *J. Nutrition*, 1943, **25**, 441.

6 Jukes, T. H., and Oleson, J. J., *J. Biol. Chem.*, 1945, **157**, 419.

7 Jukes, T. H., and Dornbush, A. C., Proc. Soc. Exp. Biol. and Med., 1945, **58**, 142.

8 Jukes, T. H., and Welch, A. D., *J. Biol. Chem.*, 1942, **146**, 19.

9 Horowitz, N. H., Bonner, D., and Houlahan, M. B., *J. Biol. Chem.*, 1945, **159**, 145.

TABLE I.
The Choline Content of Various Materials as Determined by the Reineckate and Neurospora Methods.

Material	Choline content, %	
	By reineckate	By Neurospora*
Purified diet†	<0.03	<0.04
Semipurified diet‡	<0.03	<0.04
Simplified diet§	0.04	>0.08
Peanut meal	0.21	>0.26
Range of 5 practical diets	0.10-0.13	0.11-0.15
Practical diet plus 5% liver meal	0.17	0.26

* The values preceded by the greater-than sign are lower limits of ranges having higher mean values.
† Diet 661 of McGinnis and co-workers.[3]
‡ Diet of Lucas and co-workers.[4]
§ Diet 543 of McGinnis and co-workers.[3]

indicate that, except for those compounds which are determined as choline in the reineckate method, the only compounds of significant effectiveness for Neurospora are mono- and dimethylaminoethanol and monoethylcholine.

On the simplified diet here studied, McGinnis and coworkers[3] observed that choline, methionine, and betaine prevent perosis, but on the purified diet choline only was found to be effective. They concluded that choline *per se* is required for the prevention of perosis. The results of Jukes and coworkers[6,9,10] indicate that mono- and dimethylaminoethanol and mono- and diethylcholine have choline activity for the chick. Mono- and dimethylaminoethanol may function in the chick as choline precursors since these workers found that methionine augmented materially the effects of these compounds.[10]

McGinnis and coworkers[3] have suggested that precursors of choline are present in the simplified diet on which methionine and betaine prevent perosis. Jukes and Oleson[6] have suggested that simple precursors of choline might exist in natural foods. The observations reported in the present paper support these views.

[10] Jukes, T. H., Oleson, J. J., and Dornbush, A. C., *J. Nutrition*, 1945, **30**, 219.

15482

Changes in Alkaline Phosphatase of Kidney Following Renal Damage with Alloxan.

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The alkaline phosphatase which occurs in high concentration in the proximal convoluted tubules of the kidney rapidly disappears during degenerative changes in these structures. The enzyme has been shown to be reduced by nephrotoxic drugs. Alloxan in addition to its injurious action on the islets of Langerhans causes a varying degree of parenchymatous degeneration of the renal tubules and evidence is here reported of an associated depletion of renal alkaline phosphatase in the damaged kidney.

White rats are readily susceptible to alloxan. Bailey, Bailey and Leech[1] produced

[1] Bailey, C. C., Bailey, O. T., and Leech, R. S., *New Eng. J. Med.*, 1944, **230**, 534.

in these animals necrosis of tubular epithelium with a dosage of 200 mg per kg. Dunn and McLetchie,[2] and Goldner and Gomori[3] reported somewhat similar results.

Method. Alloxan was prepared from uric acid by the method of Fischer.[4] White male rats of the Wistar strain in groups of 3 to 19 animals, after withdrawal of food for 48 hours, were injected subcutaneously with a 5% aqueous solution of alloxan in dosage ranging from 25 to 600 mg per kg of rat. Many of the rats died in 20 to 48 hours following injection of 400 mg or more per kg and younger animals did not tolerate injections as well as older ones. The rats which survived were killed by a blow on the back of the neck at 6, 21, 24, 26, 28, 30 and 48 hours after injection. Immediately after death, blood was withdrawn from the heart for glucose determination and the kidneys were quickly removed for alkaline phosphatase studies. One kidney was cut in thin slices which were fixed in Zenker's solution for routine sections, and in acetone and absolute alcohol for histochemical studies of phosphatase.

The technic used for the histochemical preparations was that of the azo dye method,[5] Gomori's method[6] and in many experiments the Kabat and Furth technic.[7] Paraffin sections of kidney tissue fixed in acetone or alcohol were sectioned at 5 μ for routine stains. Sections of both normal and pathologic tissue were affixed to the same slide and subjected to identical procedures in order to obtain comparative values.

The second kidney was used for chemical estimation of alkaline phosphatase which was carried out by a modification of King and Armstrong's[8] method as follows. The capsule was stripped from the kidney and the pelvis and medulla cut away. Duplicate portions of tissue gauged to weigh between 0.4 and 0.5 g were excised from the remaining organ, weighed exactly and each portion was ground in a mortar with alundum. Comparable duplicate results were obtained only when approximately the above amounts of kidney were used. Each lot of ground tissue was washed from the mortar into a 50 cc centrifuge tube with 100 times its weight of 0.85% sodium chloride and centrifuged 20 minutes at 2,000 r.p.m. The clear supernatant fluid was again diluted with the saline, so that the final dilution of the ground kidney was 1 to 500. The reagents required for the tests were (1) buffer substrate containing 1.09 g of disodium monophenylphosphate and 10.3 g of barbital sodium per liter of distilled water, (2) the phenol reagent of Folin and Ciocalteu[9] diluted 1 to 3 and (3) 0.1% stock phenol solution in 0.1 N HCl, the accuracy of which was controlled by titration procedures described by Peters and Van Slyke.[10]

Procedure. Into each of 4 test tubes, 4 cc of buffer were pipetted. Two of these tubes, used as duplicate controls, for the determination of preformed phenolic bodies in kidney tissue were left at room temperature and the other pair of duplicate tubes were heated to 37°C in a water bath. To all 4 tubes were then added 0.2 cc of the 1:500 dilution of ground kidney and 1.8 cc of dilute Folin-Ciocalteu phenol reagent. All 4 were heated for 30 minutes at 37°C, and then centrifuged for 7 minutes at 2,000 r.p.m. to remove protein. A 4 cc aliquot of the supernatant fluid from each tube was pipetted into 4 electrophotometer tubes and 1 cc of 20% sodium carbonate added to each at successive one-minute intervals. After the color had developed for 20 minutes they were read against

2 Dunn, J. S., and McLetchie, N. G. B., *Lancet*, 1943, **2**, 384.

3 Goldner, M. G., and Gomori, G., *Endocrinology*, 1943, **33**, 297.

4 Fischer, Emil, *Introduction to the Preparation of Organic Compounds*, trans. by R. V. Standord, Van Nostrand Co., London, 1917, 8th ed.

5 Menten, M. L., Junge, J., and Green, M. H., *J. Biol. Chem.*, 1944, **158**, 471; PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 82.

6 Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 23.

7 Kabat, A. E., and Furth, J., *Am. J. Path.*, 1941, **17**, 303.

8 King, E. J., and Armstrong, A. R., *Can. Med. Assn. J.*, 1934, **37**, 376.

9 Folin, O., and Ciocalteu, V. J., *J. Biol. Chem.*, 1927, **73**, 627.

10 Peters, J. P., and Van Slyke, D. D., *Quantitative Clinical Chemistry*, Williams and Wilkins, 655, Vol. II.

TABLE I.
Renal Alkaline Phosphatase After Alloxan Injections of 300 to 600 mg per kg Weight of Rat.

Hrs. after injection when rat killed	300 mg alloxan		400 mg alloxan		450 mg alloxan		500 mg alloxan		600 mg alloxan	
	I	II	III	II	III	II	III	II	III	II
6	158	47.5*		24.2	520	23.8	360	22.0	192	16.2
		50.6		24.1		20.8		21.2		16.2
6				22.4		21.7	208	23.8		26.3
				21.7		20.7		25.1		26.9
6				55.2			250	33.0		
				53.3				33.0		
24	174	27.4			500	13.7		12.2	375	12.8
		28.7		23.9		15.0		11.2		13.4
24			280	26.6		20.9			580	27.1
				28.5		26.0				23.9
24			580	28.9	620	29.7				
				25.0		26.0				
26				17.3			305	17.2		
				17.3				18.7		
26			580	19.5			170	16.3		
				18.7				14.9		
26			620	25.0						
				27.0						
28	133	32.6								
		31.3								
30			215		290	14.2		16.9		
				17.9		13.7		16.8		
30				38.0		19.8	224	24.8		
				36.7	580	21.1		25.8		
30			130	21.3			540	16.8		
				21.9				16.0		
48				19.2	230	22.8				
				19.2		24.1				
48				20.1						
				18.5						
Normal control	150	40.5		50.0		55.3		52.6	73	44.8
		42.0		52.3						
Normal control				46.6		58.4		62.6	93	53.8
				47.1						

I Weight of rat in mg.
II Units phosphatase in duplicate per g wet kidney.
III Blood glucose in mg %.
* Duplicate figures are included because of the fairly wide range of variation obtained in the two values.

a standard in a Fisher electrophotometer. The standard was prepared by adding 15 cc of the 1:3 dilution of the Folin Ciocalteu reagent to 5 cc of a dilute standard phenol solution (containing 0.1 mg of phenol per cc) and diluting to 50 cc. To 4 cc of this mixture was added 1 cc of 20% sodium carbonate and the color allowed to develop for 20 minutes. All tubes were read exactly 20 minutes after the addition of the carbonate. For calculation of the alkaline phosphatase the following formula was used, *viz.*

$$\frac{0.04}{RS} \times \frac{6}{4} \times 500 \times \frac{RU}{Wt} = \text{units of alkaline phosphatase in 1 g of wet kidney;}$$

where $\frac{0.04}{RS}$ = number of mg of phenol in 4 cc of standard divided by the reading of the standard on the electrophotometer, (in our experiments RS = 100); $\frac{6}{4}$ = fraction correcting for aliquot used; 500 = dilution of ground kidney; RU = colorimetric reading of unknown kidney material less the colorimetric reading of control; Wt = weight of kidney tissue used.

Results and Discussion. The general response of our animals to alloxan was similar to that reported by other workers. The intensity of the symptoms usually increased

TABLE II.
Renal Alkaline Phosphatase in Rats Dying After Alloxan Injection.

Mg of alloxan injected per kg of rat	Wt in g of rat	No. of hr after injection when rat died	Units of phosphatase (in duplicate) per g of wet kidney
300	165	48	23.9*
			23.9
400	82	25	14.7
			14.7
400	171	40	13.7
			14.2
450	169	40	14.9
			14.8
450	205	12+	15.1
			15.3
450	139	54	20.4*
			20.1
450	131	60	9.6
			9.3
450	132	120	19.2*
			19.2

* Considerable amount of blood in kidney may be responsible for high values.

with the dosage, although variations occurred in individual rats. Alloxan dosage of 100 mg or less per kg of rat produced practically no symptoms. After a dosage of 200 to 300 mg per kg the animals remained hunched up in their cages for about 24 hours but did not appear ill, and only an occasional rat succumbed to the injection. In 4 to 10 hours after the injection of 400 mg of alloxan per kg the rats appeared listless and ill. After 24 hours they were acutely ill and the excreted urine was tinted pink. Those which survived 30 hours could not stand. Those which received 500 to 600 mg per kg did not live beyond 28 hours, appeared acutely ill and their blood sugars ranged from 200 to 600 mg % at the time of death. The kidneys on excision appeared yellow or a mottled yellow and red. There was a progressive decrease in the survival rate as the dosage increased. Three of the 4 rats receiving 300 mg per kg, 14 of 19 rats of the 400 mg group and only 50% of those which received the 500 or 600 mg dosage survived.

Renal alkaline phosphatase was not decreased when the dosage was 200 mg or less per kg, as shown in 4 groups of rats, each consisting of 4 animals. Rats of each group were given subcutaneously respectively 25, 50, 100 and 200 mg per kg and killed 6, 24, 28 and 48 hours after injection. In all of the treated, as well as the normal control rats, we found the renal alkaline phosphatase to vary between 40 and 51 King and Armstrong units per g of wet kidney. Doses of 300 mg or more per kg caused an appreciable reduction in the phosphatase. The injection of 400 to 600 mg of alloxan per kg was constantly followed by a 50% or greater fall in the enzyme. With high dosage, considerable variation in individual animals in amount of residual enzyme was noted and generally the deleterious effect of the drug was enhanced with prolongation of time after injection up to 21 and 28 hours, when the maximum effect of the drug appeared to be reached. In rats surviving 2 to 3 times this period, little further diminution of phosphatase occurred. The action of the alloxan did not result in the complete removal of the enzyme because phosphatase was present in appreciable amounts in kidneys excised after death from the drug. In 4 rats dying from alloxan the residual enzyme averaged 14.7 units per g of wet kidney, a loss of nearly 70% of the total enzyme as shown in Table II. In 3 of the rats, the values higher than this may have been due to the large amount of blood remaining in these organs after death. In one animal, surviving 60 hours, the phosphatase reached a low level of 9.6 units per g of wet kidney. The water content of these organs was found to be 73 to 77% of their initial wet weight.

The reduction in renal phosphatase obtained on chemical analysis was paralleled by that found in histochemical preparations. The disappearance of the enzyme following alloxan injection could readily be detected by the naked eye in differences of color intensity occurring in microscopic sections from kidneys of alloxan treated and of normal rats when tissue sections of both affixed to the same slide were subjected to identical technic for the demonstration of alkaline phosphatase. Sections of normal kidney appeared deep gray or black, while those from treated animals were appreciably paler. The loss of enzyme occurred in the luminal border of proximal convoluted tubules and the ascending limb of the loop of Henle, areas in which the phosphatase is localized normally. Frequently in rats receiving 400 mg per kg of alloxan the phosphatase was not only diminished in the luminal border of the tubular cells but was present in large amounts in the mitochondria within the contiguous cells. Hepler, Simonds and Gurley[11] reported a somewhat similar diffusion into these cells following injury by nephrotoxic drugs. This redistribution of the enzyme was not observed with higher alloxan dosage. In a few instances the phosphatase could not be demonstrated histochemically in the proximal convoluted tubules, having been apparently, completely lost from these areas. In preparations of "Zenker" fixed tissue, with a parallel mounting of pathologic and normal sections on the same slide, which were stained with Heidenhain's iron hematoxylin stain, an augmented intensity of black stain occurred in the mitochondria in the kidneys of the alloxan-treated rats as compared to the normal animal. This finding is further evidence of the chemical change produced in the tubular cells by the alloxan. The mitochondria in the pathologic tissue appeared coarser and occasionally in sections which showed marked granular degeneration these structures also had lost their normal parallel alignment and had an irregular distribution.

Three rats which received 400 mg of alloxan per kg were given insulin on the fifth day, with a restoration of the very high blood sugar to a lower level of 200 mg %. The lowering of the hyperglycemia was not, however, accompanied by any apparent improvement in the renal alkaline phosphatase, as chemically and histochemically there was the same low level as in treated rats not receiving insulin therapy. This finding together with the fact that the diabetogenic dosage is lower than that which produces a reduction in the renal phosphatase leads us to believe that 2 different mechanisms are involved in the damage caused by alloxan in pancreas and kidney. The demonstration by Lehman[12] that alloxan in moderate concentrations inhibits the conversion of the Cori ester to the Robison ester and in high concentration sometimes inhibits the formation of both esters may indicate that the alloxan acts *in vivo* by an interference with certain enzymes which differ quantitatively or qualitatively in various tissues.

Summary. The injection of alloxan in dosage of 300 to 600 mg per kg of rat was followed by a definite decrease in the alkaline phosphatase of the kidney. With the 600 mg dosage this enzyme was reduced 50% or more. The decrease in the renal phosphatase obtained by chemical analysis was roughly paralleled by the reduction demonstrated histochemically.

11 Hepler, E. E., Simonds, J. P., and Gurley, H., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 221.

12 Lehman, H., *Biochem. J.*, 1939, **33**, 1241.

15483

Relation Between Sex Hormones and Changes in Susceptibility of Domestic Norway Rats to Alpha-Naphthyl Thiourea.*

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While investigating the acute toxicity of alpha-naphthyl thiourea (ANTU) for wild Norway rats, Dieke and Richter found that the susceptibility increases sharply at or just prior to the onset of puberty.[1] Thus, it takes about 2.5 times as much ANTU per kg body weight to kill wild Norway rats of 150 g as it does for ones of 200 g, and 6 times as much for those under 100 g. A similar change in susceptibility occurs in domestic Norway rats when thiourea is administered.[2,3] Autopsy of rats dying from thiourea or ANTU revealed massive pleural effusion and pulmonary edema. Apparently these drugs act on the lung capillaries to increase their permeability,[4] but why it should take so much more to kill young rats than adults is not known.

The experiments referred to above indicate but do not prove any direct connection between sexual maturation and susceptibility to poisoning by thiourea and ANTU. The present study, therefore, was undertaken to discover whether actual pubertal changes are responsible for this great difference in susceptibility or whether the coming into puberty at about the same time is purely coincidental. To do this, gonads were removed from one group of young rats to determine if the resistance to ANTU would remain prepubertal even after they had attained the age and weight of adult animals. A second group received sex hormone (testosterone or estrone according to the sex) in order to develop the secondary sex organs at an earlier age than normal and thus to determine whether the response to ANTU poisoning would be that of a young prepubertal rat or that of an adult.

The first part of this experiment included 40 albino rats. As it has been shown that there is no significant sex difference in resistance to acute ANTU poisoning,[1] male litter mates were used as controls for operated females and vice versa. Twenty animals were gonadectomized, the remaining 20 serving as controls. All operations were performed under ether anesthesia on either the 8th or 29th day of life (3 on the 27th). When both experimental and control animals were over 100 days old they were given intraperitoneal injections of ANTU in olive oil. The doses were adjusted so that each animal received 1/10 cc of oil suspension per 100 g body weight. Autopsies performed on all experimental animals revealed no residual gonadal tissue.

Twenty-nine experimental and 33 litter mate control animals were used in the second part of the study. One-half milligram of testosterone propionate (Perandren) was injected daily into the experimental males and 100 I.U. estrone (Theelin) in oil into the females. Injections were started from the 14th to the 22nd day of age and continued for from 10 to 19 days. At the end of the injection period both experimental and control animals were given ANTU in olive oil in the same manner as the first group. At autopsy the condition of the secondary sex organs was checked in all animals to make certain that the injected sex hormone had been effective. Rats used for these experiments came from a colony originally obtained

* This work was carried on under a contract between the Chemical Warfare Service of the U. S. Army and The Johns Hopkins University.

† On leave of absence from Hamilton College, Clinton, New York.

1 Dieke, S. H., and Richter, C. P., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 22.

2 Dieke, S. H., and Richter, C. P. *J. Pharmacol.*, 1945, **83**, 195.

3 MacKenzie, J. B., and MacKenzie, C. G., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 54.

4 Drinker, C. K., *Pulmonary Edema and Inflammation*, Cambridge, Harvard University Press, 1945, p. 39.

TABLE I.
Effect of Castration on Toxicity of ANTU in Domestic Norway Rats.

Dose (mg per kg)	No. rats	Age at castration (days)	Age on receiving ANTU (days)	Avg wt	Survival
10	3	C*	132	172	All died
8	2	8	111-128	224	1 survived
8	4	29	117-130	215	2 "
8	5	C*	106-130	191	1 "
6	3	9	127	289	All died
6	3	27	127	262	All "
6	6	C*	119-125	199	5 survived
4	3	8	128	264	2 "
4	5	29	130	194	All "
4	4	C*	127	215	" "
3	2	C*	118	287	" "

*C = Control.

from the Carworth Farms and were fed a balanced stock diet.

Table I shows the effect of prepubertal gonadectomy on ANTU susceptibility of rats at ages exceeding 100 days. No difference can be seen between those animals castrated on the 8th and those on the 29th day of age. When the data for the controls are combined an LD_{50} value of 6.7 mg/kg is obtained. It will be noted that deaths occurred among the experimental animals following similar doses. Castration therefore did not produce an increased resistance to ANTU poisoning.

Table II shows the results of the animals injected with either testosterone or estrone for a period of 10 to 19 days before administering ANTU. No significant difference was found between rats receiving the poison on the 20th day after birth and those on the 35th to 40th. All rats (with one exception) of both the experimental and control groups survived on doses of ANTU under 60 mg per kg of body weight. Among the females no difference was found between the experimental rats and their controls, animals of both groups surviving a dose of 50 mg and dying at 70 mg or more. A slight decrease in resistance to ANTU is evident in the male group receiving sex hormone, but they were still very much more resistant than normal mature animals. Among the experimental males 1 out of 4 died upon receiving 50 mg of ANTU per kg and with one exception all 12 died on doses over this. The data for the control males correspond to an LD_{50} of approximately 90 mg/kg.

Discussion. Thiourea and its derivatives presumably cause death in both young and adult rats by increasing the permeability of the lung capillaries, thus causing pulmonary edema and pleural effusion. In young animals either it requires a greater quantity of the drug to change the capillary permeability to the same degree or else the lymphatic mechanism for carrying away the fluid is more efficient in young than older rats. A comparison of the weight of the lungs with that of the body in a series of 100 normal domestic rats from our colony shows no sudden increase in the size of the lungs that might account for the formation of more fluid than the lymphatics are able to cope with. Whether or not the size and number of lymphatic channels increase at the same rate as the lung capillary bed is unknown. This paper shows that changes resulting from the presence of increased quantities of sex hormone in the body during the gradual onset of puberty are not responsible for the change in toxicity. Further investigation is in progress to determine whether other changes at this time controlled, either directly or indirectly, by the pituitary are responsible. These include growth of the animal, involution of the thyroid, and changes in metabolic rate.

Summary. In order to determine whether or not actual pubertal changes are responsible for the great decrease in resistance to ANTU observed about the time of puberty, 20 domestic Norway rats were castrated at an early age and given ANTU after reaching an age of 100 days or more. The experimental rats

TABLE II.
Effect of Sex Hormone on the Toxicity of ANTU in Domestic Norway Rats.

Dose (mg/kg)	No. rats	Sex	Age on receiving ANTU (days)	Avg wt (g)	Days of treatment ½ mg perandren per day	Days of treatment 100 I.U. theelin per day	Results
50	1	♀	21	33		10	Survived
50	2	♀	40	88		19	"
50	2	♂	35	55	19		1 "
50	2	♀	39	75		C*	"
50	3	♂	41	85	C*		"
60	2	♀	37	77		10	Died
64	1	♀	33	55		19	Survived
64	2	♀	35	54		C*	"
64	1	♂	35	58	C*		Died
70	2	♀	37	69		19	"
75	1	♀	24	37		10	"
75	2	♀	40	82		10	"
75	2	♂	24	32	10		"
75	1	♂	37	75	10		"
75	2	♂	35	52	19		"
75	1	♀	40	92		C*	"
75	1	♂	26	38	C*		"
75	5	♂	40	80		C*	Survived
100	2	♂	40	94	10		Died
100	2	♂	21	34	19		"
100	4	♂	38	71	19		2 survived
100	1	♀	38	48		19	Died
100	2	♂	26	36	C*		"
100	4	♂	41	78	C*		3 survived
100	1	♀	40	89		C*	Died
125	1	♂	40	69	10		"
125	1	♀	41	48		C*	"
125	1	♂	41	86	C*		"

* Control.

died from doses in the same range as the controls. A second group of 29 suckling rats received daily injections of testosterone or estrone. Both experimental animals and their controls were then given ANTU when they were 35-40 days old. No significant difference in the susceptibility was found. It is, therefore, concluded that the onset of sexual maturity coincides with the decrease in resistance to alpha-naphthyl thiourea but does not produce it.

15484

IV. Effect of Subtilin on the Course of Experimental Anthrax Infections in Guinea Pigs.*

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In previous communications[1,2] it was reported that subtilin showed an extremely low toxicity index to living embryonic chick heart tissue fragments cultivated *in vitro* and that it exerted a powerful *in vivo* action on the course of experimental pneumococcus Type III infections in mice. Animals treated with subtilin 9 hours after being injected with the organism were quickly cured of the infection. The antibiotic did not exhibit any apparent toxic reaction in the mice.

Subtilin is effective chiefly against Gram-positive bacteria *in vitro*.[3] A few Gram-negative organisms are equally as susceptible. However, the great majority of Gram-negative organisms are not susceptible unless considerably higher concentrations are used. *Bacillus anthracis* is Gram-positive and easily destroyed *in vitro*.

In the present communication, results are given on the effectiveness of subtilin on the course of experimental anthrax infections in guinea pigs.

Experimental. Two guinea pigs weighing approximately 500 g each were injected intraperitoneally with 0.1 cc of a 24-hour nutrient broth culture of *Bacillus anthracis*. One animal was not treated but served as the control; the other was treated immediately with 3 cc of a solution containing 2 mg subtilin per cc (total of 60 units[1]) and at stated intervals thereafter. The subtilin was dissolved in a 5% solution of glucose and all injections were made intraperitoneally. The treatments were discontinued after the 4th day and the animal kept under observation. The schedule of treatments and results are recorded in Table I.

TABLE I.
Treatment of Anthrax in Guinea Pigs.

Date	Time	Control animal	Treated animal
2-14-46	12:30 PM	0.1 cc culture	0.1 cc culture
	" "	—	3 cc subtilin
	3:30 "	—	3 " "
	6:00 "	—	3 " "
	11:00 "	—	3 " "
15	2:00 AM	—	3 " "
	4:30 "	—	3 " "
	6:30 "	—	3 " "
	9:30 "	—	3 " "
	12:30 PM	—	3 " "
	3:30 "	—	3 " "
	9:30 "	—	3 " "
16	1:30 AM	—	3 " "
	4:30 "	—	3 " "
	9:30 "	—	3 " "
	3:00 PM	—	3 " "
	10:00 "	Animal died	3 " "
17	10:30 AM		3 " "
	8:00 PM		3 " "
18	10:30 AM		3 " "
19	3:30 PM		Treatments discontinued
28	12:00 M		Animal died

It may be seen that the control animal (untreated) died in less than 72 hours. The treated animal was given subtilin for 4 days, then observed for an additional 10 days (total 14 days) when death occurred.

Since the culture of *Bacillus anthracis* contained many spores, it is believed that these must germinate into vegetative cells before they can be destroyed by the subtilin. It would appear that some spores did not germinate until after treatment was discontinued.

A second experiment was performed in which 4 guinea pigs were treated instead of only one, and the treatments continued for a longer period of time. The animals were each inoculated with 0.1 cc of a 24-hour nutrient broth culture of the organism, and varying periods of time were allowed to elapse

* This investigation was aided by a grant from Eli Lilly and Company, Indianapolis, Ind. The subtilin preparation used in these experiments was kindly supplied by the Western Regional Research Laboratory, Albany, California.

1 Salle, A. J., and Jann, G. J., Proc. Soc. Exp. Biol. and Med., 1946, **61**, 23.

2 Salle, A. J., and Jann, G. J., Proc. Soc. Exp. Biol. and Med., 1946, **62**, 40.

3 Salle, A. J., and Jann, G. J., Proc. Soc. Exp. Biol. and Med., 1945, **60**, 60.

TABLE II.
Treatment of Anthrax in Guinea Pigs.

		Animals inoculated intraperitoneally with 0.1 cc culture				
Date	Time	Control not treated	Treated immediately cc subtilin	Treated after 3 hrs cc subtilin	Treated after 6 hrs cc subtilin	Treated after 9 hrs cc subtilin
3-5	2:00 PM	—	3			
	5:00 "	—	3	3		
	8:00 "	—	3	3	3	
	11:00 "	—	3	3	3	3
6	2:00 AM	—	3	3	3	3
	5:00 "	—	3	3	3	3
	8:00 "	—	3	3	3	3
	11:00 "	—	3	3	3	3
	2:00 PM	—	3	3	3	3
	5:00 "	—	3	3	3	3
	8:00 "	—	3	3	3	3
	11:00 "	—	3	3	3	3
7	2:00 AM	—	3	3	3	3
	5:00 "	—	3	3	3	3
	9:30 "	—	5	5	5	5
	1:00 PM	—	5	5	5	5
	10:00 "	—	5	5	5	5
8	10:00 AM	—	5	5	5	5
	4:00 PM	—	5	5	5	5
	7:00 "	Dead	5	5	5	5
9	9:00 AM		5	5	5	5
	5:00 PM		5	5	5	5
10	9:30 AM		4	4	4	4
	4:30 PM		5	5	5	5
	9:30 "		5	5	5	5
11	11:00 AM		5	5	5	5
	10:30 PM		5	5	5	5
12	10:30 AM		5	5	5	5
	9:30 PM		5	5	5	5
13	9:30 "		5	5	5	5
15	3:00 "		5	5	5	5
Results		Dead	Living	Living	Living	Living

before treatments were started. The 1st animal was treated immediately after being injected with the organism; the 2nd animal was given the first treatment 3 hours later; the 3rd animal was treated 6 hours later; and the 4th animal was treated 9 hours later. The subtilin preparation used was the same as in the first experiment. All treatments were given intraperitoneally. The schedule of treatments and results obtained are given in Table II.

It may be seen that the control guinea pig died in 77 hours whereas those treated with subtilin were still living after the 10th day, when the experiment was discontinued. The guinea pig treated as late as 9 hours after being inoculated with *Bacillus anthracis* was protected from the disease. The results indicate that subtilin exerts a powerful *in vivo* action on experimental anthrax infections in guinea pigs. The antibiotic did not produce any observable toxic reaction in the animals.

Conclusions. Subtilin has been shown to produce a powerful action on the course of experimental anthrax infections in guinea pigs. Animals treated with subtilin 9 hours after being injected with the organism were protected from the infection. The antibiotic exhibited no apparent toxic reaction in the animals.

15485

Relationship Between Body Temperature and Arterial Pressure.*

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In the course of a series of investigations on the normal resting arterial blood pressures of various species of unanesthetized animals; data were obtained on the blood pressure of the turtle, *Pseudemys elegans*, at various body temperatures.

* Aided by the A. D. Nast Fund for Cardiovascular Research. The department is supported in part by the Michael Reese Research Foundation.

Arterial blood pressures were recorded on

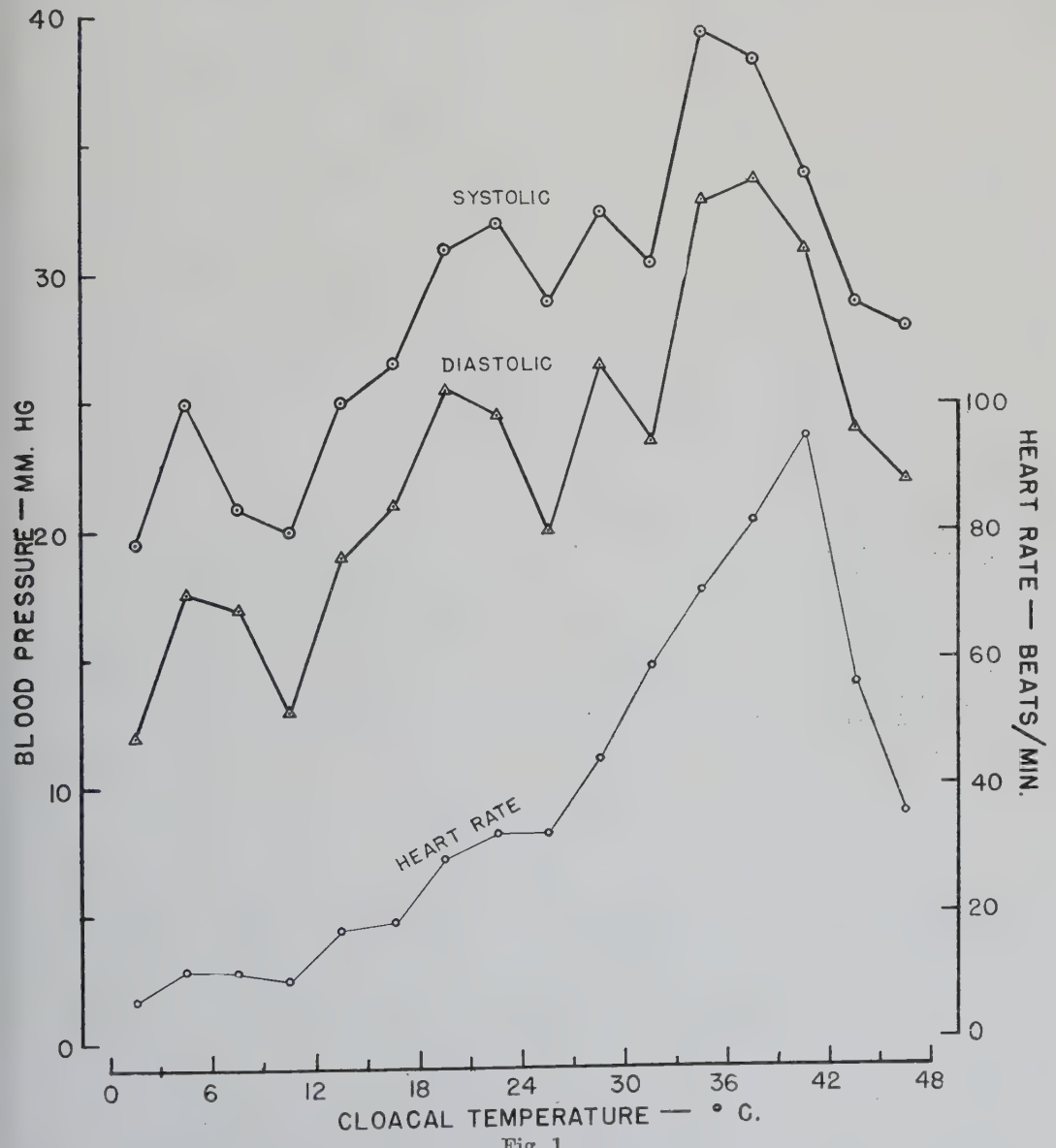

Fig. 1.

photographic paper, using the Hamilton optical recording manometer. A cannula attached to the manometer was directed cardially and tied into the left aortic arch. Body temperature was determined by means of a mercury thermometer tied into the cloaca. The animal was placed on its back in a pan of water, and the temperature of the water was changed slowly by the addition of ice cubes or by slow warming. The turtles were warmed from room temperature, 18°C, to about 40°, then cooled to about 3° and finally rewarmed to 45°. The rate of temperature change was about 0.3° per minute.

Arithmetic averages of the blood pressures and heart rates obtained in 2 heatings and one cooling of 5 turtles are given in Fig. 1. Our results show that there is a fairly consistent rise in arterial blood pressure as the cold animal is warmed until a temperature of about 38°C is obtained and with further warming the blood pressure falls rapidly. When the animals are then cooled the blood pressure increases until a temperature of about 38°C is obtained and with further cooling the blood pressure falls, so that in general the original curve is reduplicated. A second reheating gives data similar to that obtained in the first warming. The effect of temperature on heart rate is similar to that obtained with blood pressure, except that the heart rate is maximal at about 40°.

The pulse pressure remained relatively unchanged during our experiment. It decreased somewhat between 38° and 40°C when the heart rate was maximal, as can be seen in Fig. 1. It increased in 3 instances between 20° and 30°C in consequence of an unexpected fall of the diastolic pressure to zero.

Discussion. The rise in blood pressure seen with increasing body temperature may be related in some as yet undetermined way with the increased metabolic activity of the tissues. It is probably associated with an increase in cardiac output.

Comparison of our data on turtles with results on the resting blood pressure of unanesthetized mammals and birds, leads to the suggestion that there may be a general relationship between the body temperature and the level of the resting diastolic pressure. For example, the mammals (dog, rabbit, rat and man) which we have studied have all exhibited a basal diastolic arterial pressure of approximately 80 mm Hg.[1,2] These findings are in accord with scattered data found in the literature. Chickens, which have a normal body temperature of about 41°C, have a resting diastolic pressure of approximately 120 mm Hg.[3] Our data also appear to fit well with the relationship between blood pressure and body temperature in the new born rat, which has not yet attained the full development of the mechanism which assures homoiothermy.[4]

Summary. The arterial pressure of the turtle was found to vary directly with changes in body temperature between 3° and 38°C. Above 38°C the pressure declined.

1 Katz, L. N., Friedman, M., Rodbard, S., and Weinstein, W., *Am. Heart J.*, 1939, **17**, 334.

2 Rodbard, S., *Am. J. Physiol.*, 1940, **129**, 358.

3 Unpublished data.

4 Helmholz, H. F., Jr., *Fed. Proc.*, 1946, **5**, 44.

15486

Pressures Required to Produce Intradermal Wheals in Normal Human Subjects.

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A search of the literature has not disclosed any previous attempts to measure the range of pressure required to produce intradermal wheals in the normal human skin. Such pressures should be a direct measurement of the force necessary to spread the closely-bound

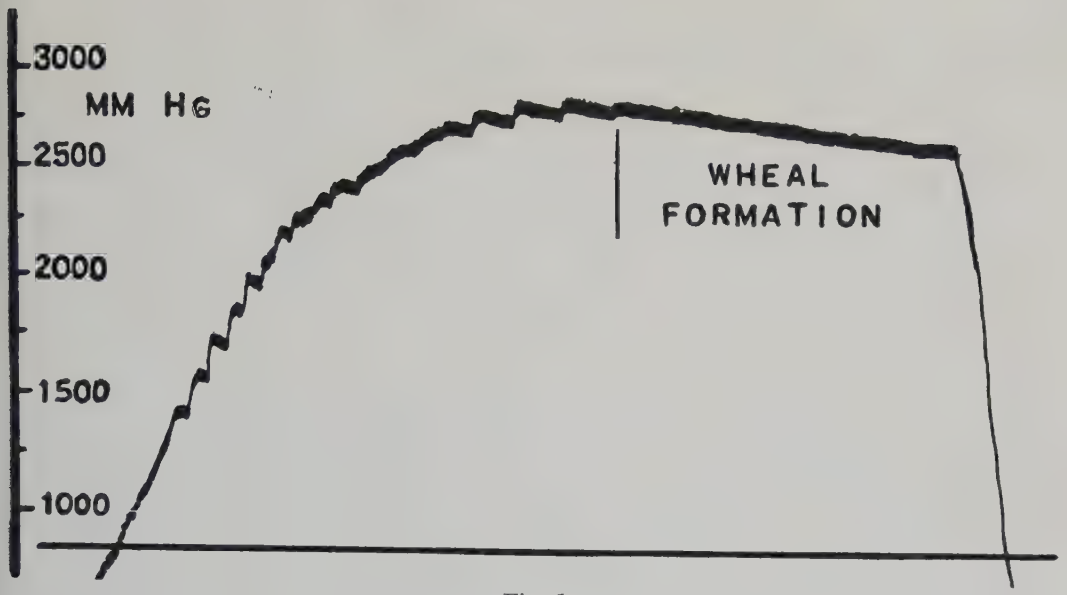

Fig. 1.

layers of the collagen connective tissue of the skin. Since natural changes may occur with varying degrees of hydration of the skin, the constancy of these pressures may be of clinical interest in such altered states as myxoedema and dehydration.

It was soon found that the pressure greatly exceeded that which could be measured by the usual Hg manometer and therefore the pressures were recorded optically. A glass spoon manometer was used with a calibration of 50 mm Hg pressure equal to 1 mm movement in the light beam which was recorded at 2½ meters on light-sensitive paper moving at a constant rate of 12 mm/second. The manometer was calibrated to 2750 mm of Hg by means of a mercury column. Wheals were raised on the lower half of the volar surface of the forearm, using a tuberculin syringe connected through a 3-way joint to the manometer and to a 27-gauge hypodermic needle ¼ inch in length. The pressure in this system was increased at a fairly rapid rate (approximately 750 mm Hg/second) until a wheal started to form. This position of the syringe plunger was then held until the wheal reached a diameter of 5 to 7 mm. To compare day-to-day variation, 5 measurements were made serially on each subject, and the average of these taken as the subject's wheal pressure for that day. Six male and 5 female subjects were used in this series.

Fig. 1 shows one of the typical pressure curves obtained. Even with this manometer it was necessary to set the zero level off the record in order to record on 60 mm paper the range of pressures involved. It should be pointed out that though we have not analyzed this part of the curve to date, the slope of the curve during wheal formation may be significant as a measure of cohesiveness of the collagen fibres of the skin.

The pressures obtained by this procedure are summarized in Table I. Considerable variation was found in consecutive readings. The average mean deviation of pressures made on the same subject on one day was 170 mm Hg with the bevel up, and 205 mm Hg with the bevel down. This is undoubtedly due to slight variations in the depth of the needle tip. Probably because of the inelasticity of the surface layers, measurements made with the bevel of the needle inserted up toward the epidermis were consistently higher than those made with the bevel down. Thus, the mean deviations on all subjects in each series and the large range of pressures in each case are not relatively as great as might be indicated by the individual figures. No consistent differences were found between

TABLE I.
The Pressures Required to Produce Intradermal Wheals.

No. of determinations	Characteristics of subjects: No.	Sex	Bevel of Needle	Solution	Pressures—mm Hg: Range	Mean pressure	Mean deviation
16	2	M	Up	Saline	1600-2800	2170	318
62	5*	M	Down	"	1000-3200	1970	454
14	3	F	Up	"	1900-3080	2240	128
24	5	F	Down	"	1280-2840	2160	400
44	4	M	"	Histamine acid phosphate 1-100,000 in saline	1260-3200	2460	405

* One Negro and one Japanese.

male and female skins, or among the skins of the Whites, Negroes, and Japanese studied. No attempt has been made in this preliminary study to determine the physiological variations in wheal-pressure which might occur with marked changes in environmental temperature or in the normal phases of the menstrual cycle. As shown in the table, the wheal-producing action of histamine is apparently too slow to lower the pressure required to produce wheals in this fashion. In fact the figures would rather indicate that histamine increased the pressure required.

Conclusion. The pressures required to produce intradermal wheals in normal individuals varied from 1,000 to 3,200 mm Hg with a mean pressure of about 2,000 mm Hg. No consistent differences in wheal pressures were found between males and females, or among Whites, Negroes and Japanese.

15487

Curative Action of Drugs in Lophurae Malaria of the Duck.*

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Scanty data have been published on the curative action of drugs in avian malarias, either in sporozoite or blood-induced infections.† No data has been published on the curative effect of drugs on lophurae malaria in the duck. For this reason, it appears advisable to record the following observations, despite the fact that the experiments are not perfectly controlled due to a large number of "accidental" deaths among our birds.‡

Infection was produced in ducks about 10 to 14 days of age by intravenous injection of 50×10^6 parasitized erythrocytes. The drug-diet method[1] of treatment was used. Administration of the diet was started 18 hours before infection and was continued for various lengths of time after infection. All drugs except SN 11,437, SN 187 and SN 475 were administered in the form of a salt.

* This investigation was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and The Johns Hopkins University.

† A considerable amount of data has been accumulated for various avian infections in the cooperative program of malarial research sponsored during the past five years by the Committee for Medical Research of the OSRD.

‡ These "accidental" deaths appear to be due to a filtrable agent. A preliminary study of this agent is reported elsewhere.[4]

[4] Dearborn, E. H., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 48.

[1] Marshall, Litchfield, and White, *J. Pharm. and Exp. Therap.*, 1942, **75**, 89.

TABLE I.
Curative Action of Drugs in Lophuræ Malaria in the Duck.

Survey No.*		Duration of treatment days	Dose mg/kg/day	No. of ducks Used	Not Cured	"Cured"	Died†
359	Quinine (base)	6	180	10	9	0	1
		10	225	9	3	1	5
390	Quinacrine, dihydrochloride	6	280-310	12	6	0	6
		13	115	9	3	0	6
971	Pamaquine (base)	6	3.5	19	6	5	8
		6	9	44	16	17	11
		10	4	10	3	1	6
7618	Chloroquine, diphosphate	6	303	5	3	0	2
		6	183	8	5	0	3
11437	N1-(5-chloro-2-pyrimidyl)-metanilamide	6	785	8	4	0	4
		26	225	7	5	0	2

* This number (SN) is used to designate a drug in the Monograph referred to above.[3]
† Figures in this column represent ducks which exhibited no evidence of infection but which died before "cure" could be established.

Subinoculations were done by intravenous injection of 2 cc of blood from the treated bird into a normal duck of about 150 g weight. Blood smears of the treated birds were made at the end of treatment, at the time of each subinoculation and as frequently as possible during the intervening periods. It was found that the injection of one to 10 parasitized erythrocytes into ducks of about 150 g weight produced an infection with the appearance of parasites in smear on the 10th-13th day. In some cases reinfection was attempted by injection of 20-30 $\times$ 10^8 parasitized erythrocytes into the treated ducks which at this time weighed about 800-1000 g. This dose in a normal duck of corresponding size would give a parasitemia of 40-50% erythrocytes parasitized on the 3rd day. The reinfected ducks were examined on the 3rd day. Those having 40% or more of erythrocytes parasitized were considered to be successfully reinoculated while those with less than 20% erythrocytes parasitized were considered to exhibit some degree of immunity.

The experiments given in this report are not as satisfactory as might be desired on account of the failure of either all the treated or all the subinoculated ducks to survive the necessary periods of observation. A series of experiments on uninfected untreated birds has shown that these deaths are not attributable to conditions of holding or procedures relevant to obtaining smears or subinoculating blood but to the filtrable contaminant mentioned in the note above. Obviously, the large number of deaths occurring before the observations are complete complicates the interpretation of the data; however, certain conclusions appear to be justified. As a result of deficiencies in our knowledge of the natural history of malaria in the duck, it must be recognized that while the presence of parasites in the blood of treated or subinoculated ducks is definitive evidence of failure to cure, inability to demonstrate either parasites in the blood or lack of immunity to them can only indicate probability of cure.

In Table I is given a very brief summary of the results obtained with the well known drugs, quinine, quinacrine and pamaquine, and also with chloroquine[2] and SN 11,437. This drug (SN 11,437) is rather unique in that it is a complete causal prophylactic in gallinaceum malaria in the chick, lophurae malaria in the turkey and cathemerium malaria in the canary.[3] However, it has no

[2] Board for the Coordination of Malarial Studies, *J. A. M. A.*, 1946, **130**, 1069.

[3] A Survey of Antimalarial Drugs, 1941-45, sponsored by the Committee on Medical Research of the Office of Scientific Research and Development (Ed. F. Y. Wiselogle).

curative effect in a blood-induced infection of lophurae malaria in the duck. Quinine, quinacrine and chloroquine are not curative, while pamaquine appears to cure a fair percentage of ducks. When treatment is started 24 hours after infection, pamaquine is still curative. However, when treatment is started 48 hours after infection a much smaller per cent of cures is produced. In the table the criterion of "cure" is interpreted as 2 negative subinoculations, the last taken at least 26 days after infection. In our experience a small percentage of ducks having 2 negative subinoculations will prove to be infected if further subinoculations are done; however, this percentage is so small that it would not significantly change these data.

In addition to the drugs listed in Table I, we have found that SN 6911, 7-chloro-4-(4-diethylamino-1-methylbutylamino) - 3-methyl quinoline; SN 1796, α-(diamylaminomethyl)-1,2,3,4 - tetrahydro-9-phenanthrene-methanol; SN 187, 3′,5′-dibromosulfanilanilide; SN 6520, 2 - (dimethylaminomethyl)-1-naphthol; SN 901, 6-chloro-2-methoxy-9-[3-(6-methoxy-8-quinolylamino)-propylamino] acridine; SN 475, 2,2′,3,3′-tetramethyl-1,1′-diphenyl-4,4′-bi-3-pyrazoline-5,5′-dione; SN 11,426, 5-(*p*-chlorophenyl) - 1-isopropyl-1-methylbiguanide, salt with acetic acid; are not curative when given in maximal tolerated dosage for 6 days. However, SN 5241, α-(dinonylaminomethyl)-1,2,3,4-tetrahydro-9-phenanthrene methanol appears to cure a certain per cent of ducks when given in the maximal tolerated dosage. With the exception of SN 5241, pamaquine is the only drug examined which appears to cure lophurae malaria in a moderate percentage of ducks.

Summary. A number of drugs have been examined for their curative action in lophurae malaria in the duck. Pamaquine appears to cure a fair percentage of the birds, while quinine, quinacrine, chloroquine and a number of other drugs do not.

We wish to thank Charlotte Kennedy, Jean Hunt, Lucille Van Ghyl, and Evelyn Epperson for technical assistance.

15488

Filtrable Agents Lethal for Ducks.*

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In studying the curative activity of drugs against lophurae malaria in ducks it was found that approximately half the birds died with no demonstrable malaria before it could be established that they were cured. Extensive studies indicated that the deaths were not due to living conditions or to the various experimental procedures to which the birds were subjected. It appeared that the causative agent was in the blood injected to produce the malarial infection. Injection of blood from normal ducks did not produce deaths.

It seemed unlikely that the lethal agent was a toxin since the deaths were delayed 15 to 30 days after infection and since the causative agent was transferable by blood passage. It also seemed unlikely that it was a form of malaria since the birds which died had no detectable erythrocytic forms of the parasite and since studies in this and other laboratories[1] had failed to reveal any large number of exoerythrocytic forms of *P. lophurae* in the duck. Bacteriologic studies indicated that none of the common bacteria was responsible for the deaths. In view of these data it seemed likely that the lethal agent might be a filtrable organism. Subse-

* This investigation was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and The Johns Hopkins University.

[1] Porter, R. J., personal communication.

quent experiments showed that if plasma from ducks with lophurae malaria were filtered through a Seitz filter impervious to *Serratia marcessans* the filtrate produced no growth in broth at 38°C but was lethal for ducks when given intravenously.

This filtrable agent associated with *P. lophurae* caused the deaths of about 50% of the birds in 12 to 30 days. Twelve to 72 hours prior to death the birds became lethargic, anorexic and weak. These symptoms became progressively more severe and terminated in convulsions followed by death. This agent was serially passed 5 times by blood transfer. In addition, it was carried with the lophurae malaria through biweekly blood transfer for at least 2 years. Passage of the malarial parasite through mosquitoes, chicks, canaries or turkeys† failed to eliminate the filtrable agent. Washing of the parasitized erythrocytes with normal duck plasma or glucose-Ringer's solution, hemolysis with 0.05% saponin and rewashing of the parasite-containing residue with normal plasma or glucose-Ringer's solution failed to eliminate the filtrable agent. This lethal effect was also associated with *P. lophurae* in ducks obtained from other laboratories. Various attempts to obtain malaria parasites, free of the filtrable agent, from ducks which had survived an infection with both also failed. A number of drugs which were tried for their curative effect in malaria had no detectable effect on the filtrable agent. Decreasing the dose of this agent decreased the percent of ducks which die. This agent was not lethal to white mice when given intravenously, intraperitoneally or intracerebrally, and it was not lethal to dogs when given in large doses intravenously.

A lethal filtrate was obtained by filtration of plasma from ducks infected with *P. cathemerium*. It was serially transferred 35 times by blood inoculation. When first obtained this agent was 100% fatal in 3 to 5 days. Its lethality was unaffected by sulfaguanidine, penicillin, sulfadiazine and a number of potent antimalarial drugs. This rapidly lethal strain was lost; however, the agent was obtained again but it was only lethal in 10 to 20 days. Various attempts to increase its virulence failed. This agent could not be washed from the parasitized erythrocytes either before or after hemolysis. Administration of the filtrate by various routes had no effect on the time of death. Apparently ducks which had survived infection with both *P. cathemerium* and this filtrable agent possessed some degree of immunity to reinfection with the filtrable agent.

Filtrates which were lethal for ducks were also obtained by Seitz filtration of plasma from ducks infected with *P. relictum* or *P. elongatum*. The former was lethal in 30 to 40 days while the latter required only 3 to 6 days. The agent associated with elongatum malaria was transferred serially 5 times by blood passage. A quantity of pooled plasma from several birds infected with this filtrate was stored at the temperature of solid carbon dioxide.

Summary. Filtrable agents, lethal for ducks, were obtained by Seitz filtration of plasma from ducks with lophurae, cathemerium, relictum or elongatum malaria. Attempts to free the malaria of the filtrable agent were unsuccessful.

† We wish to thank Dr. R. J. Porter of the University of Michigan for passing the parasites through mosquitoes, canaries, and turkeys.

15489

Immunological Similarity of Streptococcal Antifibrinolysins.*

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The development of antifibrinolytic properties in the blood of patients convalescing from streptococcal disease is commonly regarded as an immunological response to infection by the β-hemolytic streptococcus.[1-4] Measurement of the antifibrinolytic capacity of the blood has consequently been employed as a diagnostic aid in the study of infections due to this organism.[2-7]

In general, the capacity of the blood to resist the action of fibrinolysin is measured by the time required for the plasma clot to undergo complete dissolution, such tests being performed either on the patient's plasma clot[1] or on a normal plasma clot to which serum of the patient has been added.[8,9] From the results of studies with fibrinolysins from 40 different strains, Van Deventer[10] suggested that they all probably belong to one immunological type. In contrast, Mote, Massell and Jones,[11] using sharper quantitative methods, found that the resistance of plasma clots varied with fibrinolysins derived from different strains. They attributed their results to immunological differences in antifibrinolysins; the nature of these immunological differences, however, was not investigated further.

Recent investigation has indicated that antifibrinolysin is not the only factor in the blood which may confer antifibrinolytic properties on a plasma clot. Studies of the mechanism of the fibrinolytic reaction have shown that the actual lytic agent in streptococcal fibrinolysis is not fibrinolysin, but a proteolytic enzyme in the plasma (lytic factor) which is activated by fibrinolysin.[12,13] Accordingly, antifibrinolytic effects may result not only from the presence in the plasma of antifibrinolysin, but also from increased amounts of antiprotease.[14,15] In addition, it

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1 Tillett, W. S., and Garner, R. L., *J. Exp. Med.*, 1933, **58**, 485.

2 Tillett, W. S., Edwards, L. B., and Garner, R. L., *J. Clin. Invest.*, 1934, **13**, 47.

3 Tillett, W. S., *Bact. Rev.*, 1938, **2**, 161.

4 Mote, J. R., and Jones, T. D., *J. Immunol.*, 1941, **41**, 61.

5 Rantz, L. A., Boisvert, P. J., and Spink, W. W., *Science*, 1946, **103**, 352.

6 Boisvert, P. L., *J. Clin. Invest.*, 1940, **19**, 65.

7 Commission on Acute Respiratory Diseases, *J. Clin. Invest.*, 1946, **25**, 352.

8 Boisvert, P. L., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 678.

9 Van Deventer, J. K., PROC. SOC. EXP. BIOL. AND MED., 1934, **32**, 1117.

10 Van Deventer, J. K., PROC. SOC. EXP. BIOL. AND MED., 1935, **33**, 17.

11 Mote, J. R., Massell, B. F., and Jones, T. D., *J. Immunol.*, 1939, **36**, 71.

12 Kaplan, M. H., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 40.

13 Christensen, L. R., *J. Gen. Physiol.*, 1945, **28**, 363.

14 Mirsky, I. A., *Science*, 1944, **100**, 198.

15 Kaplan, M. H., *J. Clin. Invest.*, 1946, **25**, 337.

TABLE I.
Determination of Antifibrinolysin Titers in Convalescent Phase Sera by Means of Fibrinolysins from Groups A, C, and G Streptococci.

Group of infecting organism	Convalescent serum No.	Titer with Group A fibrinolysin	Titer with Group C fibrinolysin	Titer with Group G fibrinolyhin
A	100 B	83	83	83
	144 B	625	625	625
	167 B	278	278	278
	234 B	125	179	179
	463 B	179	179	179
C	120 C	83	83	56
	249 B	125	125	125
	258 C	56	56	56
	581 B	<25	25	25
	1070 C	<25	<25	<25
G	8 C	<25	<25	<25
	549 C	25	25	25
	6 C	<25	25	25
	971 D	56	56	83
	694 F	36	56	36

has been suggested that clot resistance may be due, in some instances, to a deficiency of the lytic factor in the plasma.[16,8]

Since the effect of these nonspecific antifibrinolytic factors was not controlled in previous work, the problem of the serological specificity of antifibrinolysins was reinvestigated. Antifibrinolysin was measured quantitatively by a serological method which minimized the effect of antiprotease and deficiency of lytic factor. The sera studied were obtained from patients infected by streptococci from 3 different Lancefield groups: A, C and G. In order to test for possible differences in antifibrinolysins, these sera were titrated with fibrinolysins derived from strains from each of these 3 different groups, and the resulting titers compared. The results of these studies showed that the antifibrinolysins tested in this manner were immunologically similar.

Methods. The sera tested were obtained from patients with acute streptococcal pharyngitis or tonsillitis. Streptococcal etiology was confirmed in each case by the presence of β-hemolytic streptococci in 2 or more cultures of the throat, and by the development of antistreptolysin "O" antibodies during early convalescence.[17] Streptococci were grouped by the Lancefield precipitin-tube technic[18] using bacterial extracts prepared by Fuller's method.[19] An infection was attributed to a strain of a given group if all of the throat cultures taken during the acute illness showed other groups to be entirely absent.

Antifibrinolysin levels were determined by the procedure previously described.[20] The sera were first diluted serially in 1.5-fold dilutions, beginning with a 1/25 dilution, as follows: 1/25, 1/36, 1/56, 1/83, 1/125, 1/179, 1/278, 1/417, 1/625, 1/900, 1/1400. Each dilution was incubated with a standard amount of fibrinolysin. The titer of a given serum was the highest dilution which neutralized the lysis-promoting activity of the standard unit of fibrinolysin. A difference in titer of 2 dilution increments (2 tubes), corresponding to a 2.25-fold antibody change, was considered to be beyond the error of the method.

16 Milstone, H., *J. Immunol.*, 1941, **42**, 109.

17 Hodge, B. E., and Swift, H. F., *J. Exp. Med.*, 1933, **58**, 277.

18 Swift, H. F., Wilson, A. T., and Lancefield, R. C., *J. Exp. Med.*, 1943, **78**, 127.

19 Fuller, A. T., *Br. J. Exp. Path.*, 1938, **19**, 130.

20 Kaplan, M. H., and Commission on Acute Respiratory Diseases, *J. Clin. Invest.*, 1946, **25**, 347.

TABLE II.
Determination of Antifibrinolysin Titers of Acute and Convalescent Phase Sera by Means of Fibrinolysins of Homologous and Heterologous Groups.

Group of infecting organism	Case No.	Date of serum	Antistreptolysin "O" titer	Titer with Group A fibrinolysin	Titer with Group C fibrinolysin	Titer with Group G fibrinolysin
A	O-463	1-1	159	56	56	56
		2-27	400	179	179	179
A	O-144	12-15	159	125	125	125
		1-12	317	625	625	625
A	O-167	12-21	159	83	125	125
		1-19	500	278	278	278
C	T-101	5-20	50	<25	<25	<25
		6-11	159	36	36	36
G	O-6	10-21	125	<25	<25	<25
		1-26	400	25	25	25
G	O-694	1-1	62.5	25	36	36
		2-23	100	36	56	56

The *fibrinolysins* were prepared from culture filtrates by alcoholic precipitation according to the procedure of Garner and Tillett,[21] and were stored in the frozen state until ready for use.

Results. The titers of each of 15 convalescent sera were determined with fibrinolysins derived from strains belonging to the 3 groups, A, C and G. Five of these 15 sera were obtained from patients convalescent from a group A infection; 5 were from patients with a group C infection; and the 5 remaining were from cases infected by group G strains.‡ As shown in Table I, the titer of each serum was essentially the same with each of the 3 fibrinolysins employed. Variation of the titer of any one serum did not exceed one tube. The evidence thus indicated that the 3 fibrinolysins reacted with the sera in a quantitatively identical manner.

In a second experiment these 3 different fibrinolysins were used to measure the increase in antifibrinolysin in serum specimens collected during the acute and convalescent phases of proved streptococcal infections. Sera were examined from 3 patients with group A infections, one patient with a group C infection, and 2 patients with group G infections.

As shown in Table II, the antibody rises in the group A and C cases were at least 2 tubes in magnitude; the group G cases showed increases of one tube. The amount of antibody increase in each of these patients was essentially the same with all 3 fibrinolysins employed. It was concluded that the antifibrinolysins produced in human infections due to groups A, C and G streptococci were immunologically similar.

Discussion and Summary. Several studies have indicated that a rise of antifibrinolysin apparently occurs much less frequently in streptococcal disease than a rise in antistreptolysin.[23,24,5,7] One possible explanation for this low frequency of the antifibrinolysin response is that streptococci vary markedly in their ability to produce fibrinolysin, and consequently in the frequency which they may stimulate an antibody response. Evidence has been presented in support of this hypothesis.[25,5]

21 Garner, R. L., and Tillett, W. S., *J. Exp. Med.*, 1934, **60**, 239.

‡ The detailed clinical, bacteriological, and serological data on the patients with group C and G infections are to be published elsewhere.[22]

22 Commission on Acute Respiratory Diseases, *New Eng. J. Med.*, to be published.

23 Stuart-Harris, C. H., *Brit. J. Exp. Path.*, 1935, **16**, 513.

24 Winblad, S., *Acta Path. et Microbiol. Scand.*, 1941, Supp. 44, 1.

25 Commission on Acute Respiratory Diseases and Kaplan, M. H., *Science*, 1945, **101**, 120.

However, a second explanation was suggested by the report[11] that antifibrinolysins differed immunologically. If antibody differences were a significant factor in the determination of antifibrinolysin, it seemed possible that the detection of antibody rises might be limited to the variety of fibrinolysin employed as antigen. The present study was consequently undertaken to test for and, if possible, to determine the nature of the differences in antifibrinolysins.

An attempt was made to obtain evidence of such differences by measuring the antibody level of the sera of patients infected by streptococci belonging to different Lancefield groups. Quantitative antibody titrations were carried out on the sera of patients with infections due to organisms from each of the 3 groups, A, C and G; and the fibrinolysins used for testing for antibody were derived from strains from each of these same groups. The results of the study showed that the titers of the various sera tested were essentially the same with all 3 fibrinolysins.

It would thus appear that the fibrinolysins produced by these different organisms were immunologically identical in their reactivity. This is in agreement with the observations of other workers.[3,10,26] It is not possible to explain the different results reported in the first quantitative studies of the problem.[11] However, as possibly contributing to apparent differences, 2 points may be mentioned: (1) the nonspecific factors in the plasma, such as antiprotease or a deficient lytic factor, may have produced the differences observed, or (2) the variations may have been due to the method of antibody estimation employed. Measurement of antibody was based on the amount of fibrinolysin which permitted clot dissolution. Since fibrinolysin and antifibrinolysin combine in varying multiple proportions,[15] it would appear that a procedure in which the fibrinolysin concentration is varied is not suitable for the measurement of antibody. The evidence thus suggests that the fibrinolysins produced by streptococci pathogenic for man are identical in their immunological behavior.

Conclusion. It was concluded, therefore, that the use of a single fibrinolysin preparation is satisfactory for the serological determination of antifibrinolysin in human infections.

26 Kirby, W. M., and Rantz, L. A., *Arch. Int. Med.*, 1943, **71**, 620.

15490

Action of Estrogen on Release of Hypophyseal Luteinizing Hormone.

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The gonad stimulating properties of the pituitary are better understood than is the reciprocal action of the gonadal secretions on the pituitary. In respect to the latter process the terms "suppression" and "inhibition" are frequently used to describe what is obviously a more complicated mechanism than these empirical terms imply. The pituitary gonadotropic complex does not always act as a unit and individual factors should be considered separately in their response to estrogens and androgens. Furthermore, the intracellular synthesis of a pituitary factor is a process distinct from that involving the liberation of the factor from the gland.

We have attempted in the work presented here and in our previous studies,[1,2] to obtain

1 Hellbaum, A. A., and Greep, R. O., *Am. J. Anat.*, 1940, **67**, 287.

2 Hellbaum, A. A., and Greep, R. O., *Endocrinology*, 1943, **32**, 33.

TABLE I.

Comparison of the Residual Pituitary Gonadotropic Activity Found in: Normal Adult Female Rats; Female Rats 3 Months After Spaying; Spayed Female Rats Treated with Estrogen for 30 to 45 Days Beginning at 3 Months After Spaying.

Adult female donors			Immature recipient female rats			
				Ovarian wt		
Type	No. of animals	Avg dose (mg)	No. of animals	Avg (mg)	Range (mg)	Type of ovarian response
Normal 21-day-old female recipients.						
Intact	38	14.5	7	29.1	16- 49	2 to 5 corpora lutea
Spayed 3 months	8	3.6	8	73.9	32-135	Numerous corpora lutea
	6	1.7	12	39.0	23- 54	" " "
Spayed 3 months then treated with 15μ estradiol benzoate 30 to 45 days	14	13.8	7	31.3	20- 42	6 rats with follicles only 1 rat with corpora lutea
Avg ovarian wet of uninj. immature control rats—12 mg.						
Hypophysectomized Female Recipients						
Intact	40	19.2	5	27.2	21- 34	2 to 5 corpora lutea
Spayed 3 mo	9	3.54	9	54.8	34-103	Numerous corpora lutea
	5	1.7	10	32.4	19- 56	" " "
Spayed 3 mo then treated with 15μ estradiol benzoate 30 days	7	6.4	7	20.2	16- 28	Follicles only
Spayed 3 mo then treated with 3μ estradiol benzoate 30-45 days	9	5.8	9	15.4	9- 26	" "
Avg ovarian wgt of 8 hypophysectomized immature controls—8.7 mg.						

information on the storage and release of pituitary gonadotropins. The purpose of this report is to demonstrate the qualitative changes in the residual luteinizing component of the pituitary at various levels of estrogen influence.

The experimental procedure consisted of comparing the type of ovarian stimulation produced by the pituitaries of: (1) normal adult female rats; (2) adult female rats 3 months after the removal of the ovaries; (3) oophorectomized adult female rats injected with estradiol benzoate after a 3-month postoperative interval. The pituitaries of the different donor groups were assayed in normal female rats 21 days of age and in immature hypophysectomized female recipients.

The donor pituitaries, following removal, were dehydrated in acetone, dried, and powdered by the procedure previously reported.[1] The powder was suspended in water and injected twice daily for 3 days into normal and hypophysectomized recipients. The latter received their first injection 48 hours after the operation. Necropsies were made 120 hours after the first injection. The ovaries were weighed and examined before fixation for follicle growth and for presence of corpora lutea. Histological sections were prepared when corpora lutea were not grossly visible. The data are summarized in Table I.

Normal Adult Female Rats. Assays carried out on the pituitaries of this group revealed the characteristic gonad stimulating activity of pituitaries which have been subjected to the normal output of ovarian hormones. A total of 78 glands were assayed in 7 normal and 5 hypophysectomized recipients (Table I). The resultant ovarian responses showed that the pituitary gland

of the normal rat contains moderate amounts of the luteinizing hormone. There were from 2 to 5 corpora lutea in the ovaries of each of the recipients.

Spayed Adult Female Rats. In this group, 28 adult female rats were oophorectomized 3 months prior to necropsy and removal of the pituitary glands for assay. A total of 20 normal and 19 hypophysectomized recipients were used. The pituitaries from this donor group showed a marked increment in the luteinizing factor. The ovaries of the recipient rats were extensively luteinized. Macroscopic and histologic examination revealed ovaries filled with corpora lutea and few, or no maturing follicles.

In addition to an increase in the residual luteinizing factor, following spaying, the pituitary content of follicle-stimulating hormone was likewise increased. Less than 1/10 of the dose of pituitary powder from spayed rats was required to produce an ovarian weight increase comparable to that produced by the pituitaries of normal animals. This was undoubtedly due, in part, to an increased storage of the luteinizing hormone which acted synergistically with the follicle-stimulating hormone to produce greater ovarian enlargement.

Oophorectomized Adult Female Rats Injected with Estradiol Benzoate. The object of this experiment was to determine whether the store of luteinizing hormone, which had accumulated in the pituitary as a result of spaying, could be released by estrogen injection and be reduced to such a degree that its physiological activity would be undetectable in recipient female rats.

A total of 21 adult animals, which had been spayed 3 months previously, were injected daily with 15 μ estradiol benzoate* in oil for 30 to 45 days. The pituitaries of these animals were tested in 7 intact and 7 hypophysectomized recipients. The ovaries of only one of the assay animals contained corpora lutea. The ovaries of the remaining 13 animals showed only follicle stimulation. The presence of lutein tissue in the single intact recipient may have been due to endogenous luteinizing hormone from the test animal's own pituitary gland.

Another group of 9 adult oophorectomized rats were injected with 3 μ of estradiol benzoate daily for periods varying from 30 to 45 days. These pituitaries were administered to 9 hypophysectomized recipients. The ovaries of none of these animals showed a trace of luteinization, although most of them had well developed follicles.

It is clear from these data that estrogen caused a liberation of the luteinizing hormone to a point where this substance was being released as rapidly as it was being produced. This finding is in accord with our previously expressed views regarding the effect of the gonads on the pituitary. Although our former study[2] was concerned primarily with the action of testosterone, we doubt that there is an essential qualitative difference between the action of estrogen and testosterone on the pituitary with respect to their ability to cause a release of the luteinizing hormone. This view concurs with the findings of other workers investigating functional activity of the pituitary, with respect to gonad stimulation, during treatment with sex hormones.[3-7] Laqueur and Fluhmann[8] injected testosterone into normal female rats and obtained a diminution of gonadotropic potency (by implants) which they attributed to an inhibition of the production of luteinizing hormone.

Summary. The pituitaries of adult female rats, under normal estrogen influence, stimulated moderate luteinization in normal and hypophysectomized recipient immature female rats. The pituitaries of adult female rats freed of estrogen stimulation through oophorectomy, invariably caused extensive luteinization of the recipient ovaries. This

* The estradiol benzoate was kindly furnished by the Schering Corporation, Bloomfield, N.J.

3 Hohlweg, W., *Klin. Wchnschr.*, 1934, **13**, 92.

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5 Wolfe, J. M., and Hamilton, J. B., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 189.

6 Fevold, H. L., Hisaw, F. L., and Greep, R. O., *Am. J. Physiol.*, 1935, **114**, 508.

7 Freed, S. C., Greenhill, J. P., and Soskins, S., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 440.

8 Laqueur, G. L., and Fluhmann, C. F., *Endocrinology*, 1942, **31**, 300.

indicated that liberation of the hypophyseal luteinizing factor was minimal in the absence of estrogen; the factor remaining stored within the pituitary gland.

Release of the luteinizing factor by estrogen was suggested also by the type of ovarian response produced by pituitaries from oophorectomized adult female rats which had been injected with estradiol benzoate daily for 30 to 45 days. These pituitaries produced follicular development but no corpora lutea, due to removal of the luteinizing factor by the estrogen treatment.

15491 P

Effect of Long Chain Fatty Acids on Bacterial Growth.

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We have shown elsewhere that certain water soluble lipids promote diffuse growth of tubercle bacilli in synthetic media, especially in the presence of serum albumin.[1,2] These same substances have now been found to enhance the growth of other microbial species, in particular of an unidentified micrococcus (strain C), recently isolated in our laboratory. Some understanding has also been gained of the conditions under which long chain fatty acids can stimulate bacterial growth.

It is known that the soaps of fatty acids exert a bacteriostatic and bactericidal effect on certain micro-organisms, particularly on the Gram-positive and acid-fast species, and that unsaturated acids are more toxic than the corresponding saturated compounds.[3-9] For example, concentrations of oleic acid as low as 0.000001-0.00001% are sufficient to cause inhibition or retardation of growth of small inocula of human tubercle bacilli in synthetic liquid media. On the other hand, fatty acid esters (methyl oleate, triethanolamine oleate, phosphatides) exhibit little or no primary toxicity. That the lack of toxicity is not due to poor solubility of the esters is indicated by the fact that the polyoxyethylene derivatives of oleic acid are essentially nontoxic, even though they are completely dispersible in water.[1,2] Thus, tubercle bacilli grow readily in synthetic media to which has been added 0.1-1.0% of Tween 80 (a polyoxyethylene derivative of sorbitan monooleate) purified to remove unesterified fatty acid.[10] Detoxification of the fatty acids can also be achieved by adding to the medium native serum albumin. When an adequate amount of this protein is added to an opalescent soap emulsion (at neutral pH), there occurs an immediate clearing of the emulsion with concomitant disappearance of toxicity. It takes approximately 40 parts by weight of albumin to achieve complete detoxification of 1 part of oleic acid; however, growth of tubercle bacilli can be obtained in media containing 0.01% oleic acid and 0.5% serum albumin if a sufficiently large inoculum is used.

When rendered nontoxic, either by ester-

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3 Avery, O. T., *J. Am. Med. Assn.*, 1918, **71**, 2050.

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TABLE I.
Effect of Oleic and Stearic Acid on Bacterial Growth.

Added to casein hydrolysate medium				Growth in mg/10 ml*—7 days incubation	
Glucose %	Oleic acid %	Stearic acid %	Albumin %	Micrococcus C mg	Avian tubercle bacillus mg
0	0	0	0	0.2	0.1
0	0.001	0	0	1.0	0
0	0.01	0	0	0	0
0.5	0	0	0	0.3	0.1
0.5	0.001	0	0	2.1	0
0.5	0.01	0	0	0	0
0	0	0	0.5	0	0.2
0	0.001	0	0.5	1.0	0.3
0	0.01	0	0.5	3.9	1.1
0.5	0	0	0.5	0	0.3
0.5	0.001	0	0.5	4.1	0.5
0.5	0.01	0	0.5	5.1	1.1
0	0	0.001	0.5	0	0.3
0	0	0.01	0.5	0	0.8
0.5	0	0.001	0.5	0	0.6
0.5	0	0.01	0.5	0	1.2

* The amount of growth was evaluated from measurements of optical density and of sediments obtained by centrifugation in comparison with suspensions containing known weights of bacteria.

ification or by admixture with serum albumin, a number of long chain fatty acids are found able to enhance the growth of certain bacteria; the different bacterial species, however, differ markedly in their response to the various acids. The comparative behavior of tubercle bacilli and of micrococcus C in this respect can be summarized in the following statements.

Enhancement of growth of tubercle bacilli can be obtained by adding 0.01% of any of a variety of long chain fatty acids—saturated or unsaturated—to a medium containing 0.5% crystalline serum albumin. The presence of glucose or of other readily available carbon compounds is not necessary for the development of growth or for demonstration of the enhancing effect of the fatty acid. This finding is in agreement with the fact that several long chain fatty acids have been found to stimulate oxygen uptake by tubercle bacilli.[11] In the case of micrococcus C, on the other hand, abundant growth results from the addition of oleic, linoleic, linolenic or arachidonic acids (0.0001-0.001%) to a mineral medium containing glucose as the sole source of carbon. Saturated fatty acids, on the contrary, appear completely unable to permit growth. In this respect the nutritional requirements of micrococcus C are similar to those of diphtheria and tetanus bacilli.[12,13] Although none of the other substances tested (yeast extract, hydrolysates of casein, protein, etc.) can take the place of the unsaturated fatty acids in initiating growth of the micrococcus, the abundance of the growth is markedly increased by the addition of glucose to a medium containing an adequate concentration of unsaturated acid. Finally, addition of crystalline albumin to media containing only minute concentrations of unsaturated acids completely inhibits the small amount of growth of micrococcus which would have taken place in the absence of the protein; this inhibitory effect can be neutralized by adequate addition

[11] Loebel, R. O., Shorr, E., Richardson, H. B., *J. Bact.*, 1933, **26**, 139.

[12] Cohen, S., Snyder, J. C., and Mueller, J. H., *J. Bact.*, 1941, **41**, 581.

[13] Feeney, R. E., Mueller, J. H., and Miller, P. A., *J. Bact.*, 1943, **46**, 559.

of oleic, linoleic, linolenic or arachidonic acid. No similar growth inhibitory effect by albumin has been observed in the case of tubercle bacilli.

These different phenomena are illustrated in Table I in which are compared the growths of a strain of avian tubercle bacilli and of micrococcus C in a casein hydrolysate medium to which oleic acid, stearic acid, glucose and serum albumin were added as indicated.

More extensive data concerning the effect of different fatty acids on bacteria—both in liquid and on agar media—will be presented in a forthcoming publication; it will be shown also that, at equal concentrations of long chain fatty acids, the water soluble esters are more efficient than the corresponding soaps in supporting bacterial growth.

It appears worth pointing out at this time that, under the proper cultural conditions, the amount of growth yielded by micrococcus C seems to be directly related to the amount of unsaturated fatty acids present in the medium (between 0.00001 and 0.0001%). This property suggests that the culture might lend itself to the development of a microbiological assay method for these lipids.

15492

Heart Rate of the Albino Rat.*

LOUIS E. MOSES. (Introduced by F. E. Emery.)

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Hoskins, Lee and Durrant[1] counted the heart rates of rats by stethoscope while the animals lay dozing in their cages, and they reported a basal rate of 281±18 beats per minute. Fishburne and Cunningham[2] wrapped their rats in a towel for auscultation of the heart sounds; the rates they obtained fell in the neighborhood of 300 beats per minute. Meyer and Yost[3] held their rats in the crook of the arm while palpating the apex beat, and they found the rates of adult rats to vary between 300 and 330 beats per minute.

Drury, Harris and Maudsley,[4] Robertson and Doyle,[5] and Leblond and Hoff[6] recorded electrocardiograms in rats and thus avoided subjective errors, but they did so while forcibly restraining their rats in the supine position to allow insertion of needle electrodes through the skin. Kniazuk[7] described an oscillographic method for obtaining electrocardiograms from rats placed in specially constructed cages. The method developed independently by the present author follows the same principle as that of Kniazuk but has the advantage of making inexpensive permanent records. It involves registration of the electrocardiogram from the surfaces of the rat's feet.

At first, the record was transcribed with the usual electrocardiograph, but, aside from the cost of bromide paper and the time lost in developing the film, that procedure was unsatisfactory in that it failed to tap the heart's potential through plantar callouses that the rats acquired in their mesh-floored cages.

The apparatus finally adopted was that developed for the recording of brain waves

* Publication No. 596, research series, the University of Arkansas.

1 Hoskins, R. G., Lee, M. O., and Durrant, E. P., *Am. J. Physiol.*, 1927, **82**, 621.

2 Fishburne, M., and Cunningham, B., *Endocrinology*, 1938, **22**, 122.

3 Meyer, A. E., and Yost, M., *Endocrinology*, 1939, **24**, 806.

4 Drury, A. N., Harris, L. J., and Maudsley, C., *Biochem. J.*, 1930, **24**, 1632.

5 Robertson, E. C., and Doyle, M. E., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 139.

6 Leblond, C. P., and Hoff, H. E., *Am. J. Physiol.*, 1944, **141**, 32.

7 Kniazuk, M., *J. Lab. Clin. Med.*, 1937, **22**, 868.

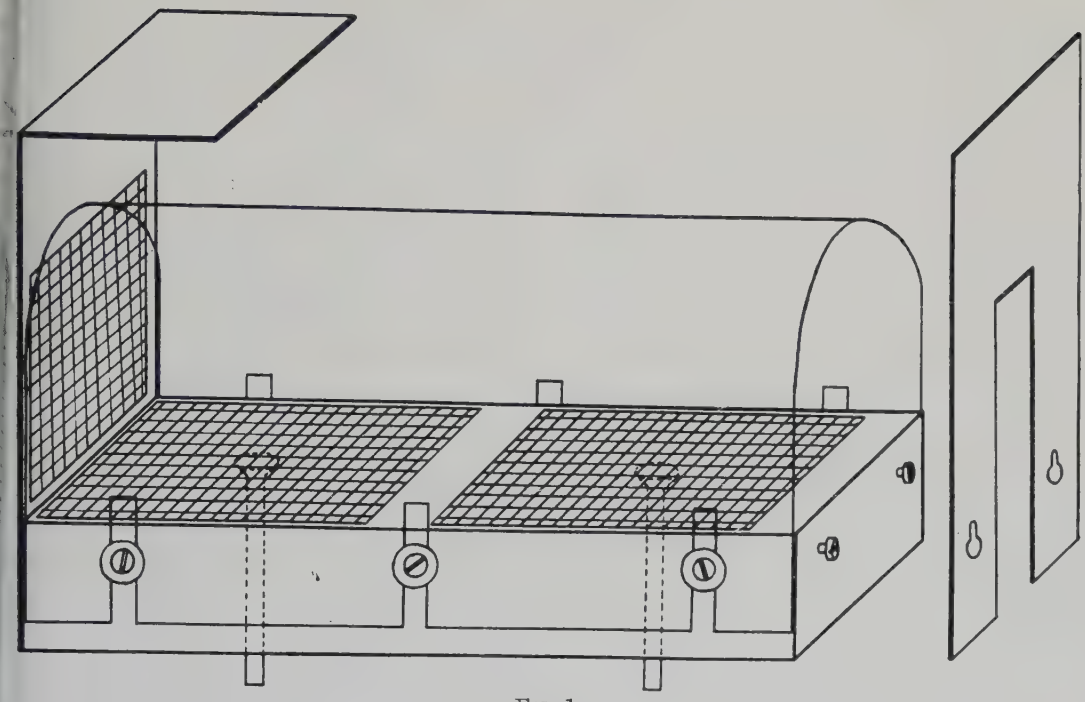

FIG. 1.
Rat holder for heart rate determination. Approximately one-fourth actual size. Description in text.

by Gerard and his associates. In this method, the impulse is picked up by contact electrodes in the form of copper floor plates, stepped up by Offner's[8] 3-stage push-pull amplifier, and inscribed on adding machine tape by the piezo-electric crystograph of Offner and Gerard.[9] Since the tape is moved by a constant speed motor, the heart rates can be measured accurately by the use of dividers.

Construction of the animal holder is shown in Fig. 1. The floor is a 1″ x 2½″ x 6″ hard rubber block into which copper plates are countersunk to act as the pick-up electrodes, one for both forefeet and the other for the hind feet. To simulate the rough floor to which rats are apparently partial, a square of copper screening is soldered to the surface of each of the copper plates. A long iron screw, soldered to each plate, passes through the hard rubber floor to dip into a mercury cup set into the shelf on which the cage rests. The potentials are led from these mercury cups into the amplifier and crystograph. Animal, cage, and wiring are shielded from 60-cycle hum by enclosure in a large copper screen chamber, appropriately grounded.

Neither electrode paste nor saline is required to facilitate transmission from the rat to the electrodes on which he is standing.

To make each cage adjustable for rats of all sizes, the sides and roof are formed by a single bent sheet of heavy celluloid, measuring 7″ x 6″. Slots cut into the celluloid sheet in place of screw holes thus make it possible to alter the volume of the cage at will.

The front gate is constructed of heavy sheet metal, 2¾″ x 6½″, with a large copper screen window for ventilation. The top of this front gate is bent to form a 2-inch projection to shade the animal's head. (This author has found no merit to the almost universal assumption that bright light induces rats to sleep; it probably irritates their eyes,

[8] Offner, F., *Rev. Sci. Instruments*, 1937, **8**, 20.
[9] Offner, F., and Gerard, R. W., *Science*, 1936, **84**, 209.

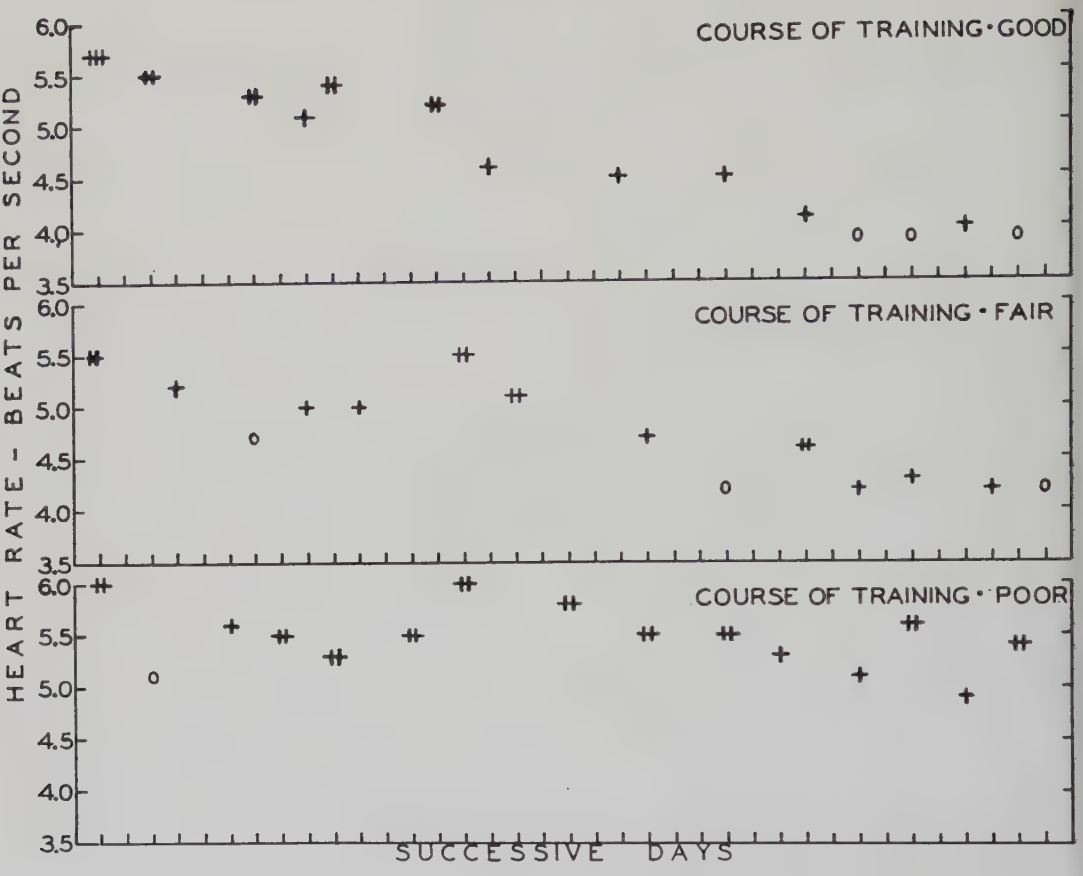

FIG. 2.

Decline in heart rate with progressive training. Evaluation of activity: ++++, extreme and prolonged; +++, intermittent but great; ++, moderate; +, occasional and mild; 0, insignificant; ∞, prolonged inactivity.

which are unprotected by pigment. Actually, it was observed that coating the shiny front screen with flat black paint aids significantly in producing relaxation).

The tail gate is also of heavy sheet metal, its dimensions 2¾″ x 4″. A large slot is cut into this gate, from below upward, to allow protrusion of the tail and testicles. A small plate is used to close this slot in those instances, early in the training period, when a rat succeeds in turning around in the cage; without such a device, he is likely to gnaw at the edge of the tail gate.

For the most part, adult male rats have been used in this study, and most of the observations regarding heart rate levels and training regimes were made upon them. The training was facilitated by use of a battery of heart rate cages, since a preliminary rest of 15-20 minutes was found necessary before the heart rate reached basal levels. Generally, the records taken for experimental purposes, after training, were made on each rat separately, involving a 10-minute run following a 10-minute rest. Later, it was found that a restless animal could be prevented from communicating his mood to a neighbor by interposing a partition between adjacent cages. With this modification, a number of rats can be rested simultaneously and then each cage switched in sequence into the circuit for recording.

The heart rate adopted from any given run is the lowest one found in that tracing. The trend of succeeding determinations is followed from the curve of daily findings; that value indicated by a leveling off of the curve is taken as the "predictable,"

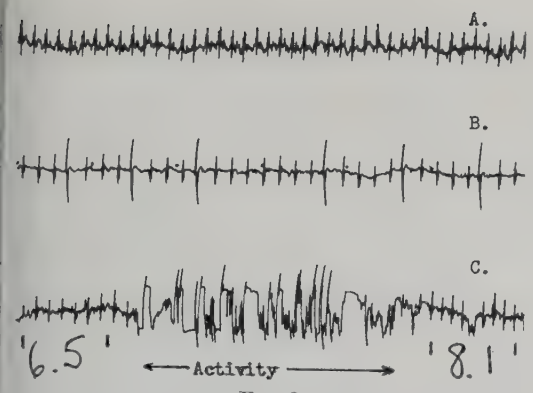

FIG. 3.

Illustrative tracings. Approximately one-half actual size.

a. Fairly complete ECG.

b. Ventricular extrasystoles (large, premature QRS complexes).

c. Effect of activity on heart rate (early phase of training).

if not always true basal, heart rate. The resultant values on 113 adult males, ranging from 200 to 370 g body weight, averaged 4.4±0.30 beats per second, or 264±18 per minute.

The attainment of a predictable heart rate level usually was found to require at least a month of training.

The course of training is fairly consistent in practically all instances, as indicated by 3 curves selected to bring out the average and the 2 extremes (Fig. 2). In general, the rats become progressively more and more

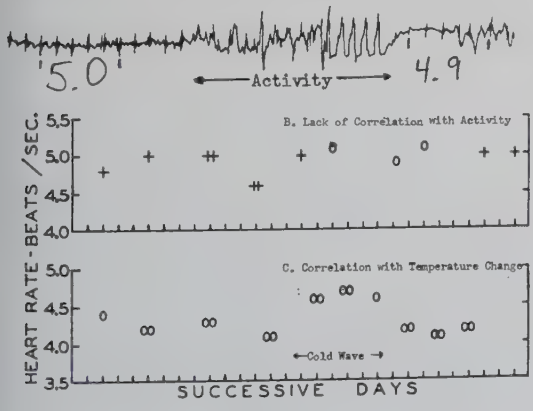

FIG. 4.

Factors altering heart rate.

a. Lack of effect of activity (late phase of training period).

b. Lack of day-to-day correlation with activity.

c. Effect of room temperature.

accustomed to the regime, and, though only a rare individual goes to sleep in the cage, most come to accept the restriction with equanimity. The symbols used on the curves are a purely subjective evaluation of activity as determined by inspection of the tracings.

The tracings obtained do not usually constitute complete electrocardiograms. A fair proportion of them do show the customary 3 major deviations of the cardiac cycle (Fig. 3a), but most record only a triphasic QRS complex. No doubt, establishment of better contact between the feet and the electrodes would suffice in all instances to bring out the P and T waves, if desired.

Occasionally, an individual rat exhibits cardiac arrhythmia, varying from a slight irregularity to a series of extra-systoles of apparently ventricular origin (Fig. 3b). In none of these animals was there noted any other sign of impaired health.

Any bodily activity is marked by large, irregular oscillations of the crystograph pen and so is easily distinguished on the tracing. In general, the heart rate varies with the degree and violence of movement (Fig. 3c).

However, it soon became clear that physical activity is not the only factor causing variations in heart rate, nor is it the most important factor. In a large proportion of subjects, neither the "basal" rate of an individual tracing nor the rates obtained on different days jibe as completely as one might predict with the state of rest or activity (Fig. 4b). Since a spontaneous quickening of the pulse often precedes a movement and since, in other instances, activity is neither accompanied nor followed by cardio-acceleration (Fig. 4a), it seems that greater variance is induced by the state of the emotions than by that of the musculature. The relative importance of this factor is further stressed by the uniform finding that increases in heart rate with body movement are of great degree only during the early phases of the training period.

Temperature changes likewise exercise an important influence on the heart rate. Much of this study was carried out in a constant temperature room at the University of Chi-

cago, and occasionally in wintertime, during a severe cold wave, the room temperature declined by several degrees. All heart rates taken during such periods were greatly elevated, even in rats which were resting quietly (Fig. 4c). In the investigation for which these heart rate studies were made,[10] one highly effective method used to accelerate the heart was chilling of the animal by depilation.

From these evidences it seems clear that dependable normal pulse rate determinations can be made in the unanesthetized rat, providing the extreme excitability of the animal and the great variability with temperature changes are recognized and steps taken accordingly. Early indications of studies in progress are that the training period may be cut to a week or two by the use of roomier cages than were formerly deemed necessary.

Summary. 1. A method is presented for accurate determination of the normal, conscious, resting heart rate in the albino rat. 2. The average value obtained on 113 adult males of 200-370 g body weight was 4.4 ± 0.30 beats per second, or 264 ± 18 per minute. 3. Emotional states were found to cause greater variation in heart rate than muscular activity. 4. Alterations in environmental temperature proved to exert a decided influence on the rat heart rate.

[10] Moses, L. E., *Am. J. Physiol.*, 1944, **142**, 686.

15493 P

Effect of Alloxan upon External Secretion of the Pancreas.

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The report of Goldner and Gomori[1] that in dogs made "diabetic" by alloxan injection the intralobular duct cells of the pancreas showed vacuolization suggested to us the possibility that this might serve as a means of investigating whether these duct cells perform a secretory function.

Methods and Results. A standard preparation of secretin concentrate designated S_1[2] was used in these experiments. It is known to contain both secretin and pancreozymin. The particular lot of material used for these experiments was tested on 2 normal dogs and the threshold dose[3] was found to be 0.3 and 0.5 mg respectively, indicating that the material had standard potency. In 4 dogs rendered "diabetic" for 18 to 30 days by the intravenous injection of 75 mg of alloxan per kg of body weight, the threshold dose of this same lot of secretin concentrate was found to be 2, 4, 5 and 10 mg. The "diabetic state" was attested by the strongly positive sugar reaction of the urine accompanied by moderate decline in body weight.

Two dogs which were similarly treated with alloxan but failed to become "diabetic" showed a threshold of 0.4 and 0.8 mg secretin concentrate, respectively. In the 2 normal dogs mentioned above, which were used to establish the potency of the secretin preparation, the injection of alloxan in a dose of 75 mg per kg of body weight after the threshold response had been determined did not significantly alter their response to a subsequently administered threshold dose of secretin concentrate within 3 hours.

Amylase determinations by the method of Schmidt[4] were performed on all samples of

* Present address: University of Illinois College of Medicine, Chicago.

[1] Goldner, M. G., and Gomori, G., *Endocrin.*, 1943, **33**, 297.

[2] Greengard, H., and Ivy, A. C., *Am. J. Physiol.*, 1938, **124**, 427.

[3] Ivy, A. C., Kloster, G., Drewyer, G. E., and Lueth, H. C., *Am. J. Physiol.*, 1930, **95**, 35.

[4] Schmidt, C. R., Greengard, H., and Ivy, A. C., *Am. J. Digest. Dis.*, 1934, **1**, 618.

TABLE I.
Secretin Threshold Dose and Amylase Concentration of Pancreatic Juice in Control Dogs and Dogs Treated with Alloxan.

Dog No.	Alloxan (mg/kg)	Days after alloxan	Urine sugar	Amylase (mg glucose/cc)	Threshold dose of secretin (mg)
1	none	—	0	1460	0.3
2	,,	—	0	1280	0.5
3	75	18	++	1760	2.0
4	75	24	++	1120	10.0
5	75	28	+++	1690	5.0
6	75	30	++	1430	4.0
7	75	22	0	1260	0.4
8	75	30	0	1740	0.8

pancreatic juice but revealed no remarkable differences between the juice obtained from alloxan-treated animals and that of normal dogs (Table I). In one animal with alloxan-induced diabetes in which the pancreas was examined histologically, the vacuolization of the intralobular ducts described by Goldner and Gomori[1] was found; the acinar cells were normal.

Discussion. The finding of decreased responsiveness to secretin accompanied by histological evidence of damage to the duct epithelium[1] suggests but does not prove that the small duct cells participate in the formation of pancreatic juice. It is generally taught that the entire pancreatic juice is produced by the acinar cells; no secretory function is ascribed to the duct cells. In the salivary glands the duct cells are believed to contribute most of the liquid portion of the saliva.[5]

Two separate hormones, namely secretin and pancreozymin, control the secretory activity of the pancreas. The type of activity engendered by each hormone (water and bicarbonate secretion in the case of secretin; enzyme secretion in the case of pancreozymin) can vary independently. These facts are compatible with the concept that each hormone acts predominantly upon a different cell type in the pancreas, the secretin upon the intralobular duct cells and the pancreozymin upon the acinar cells.

A similar situation obtains in the gastric glands where the parietal cells produce hydrochloric acid and most of the water of the gastric juice while the granulated body chief cells provide the pepsin. The analogy between the gastric and pancreatic glands can be extended to include a functional parallelism, *to-wit,* the relationship between the rate of secretion of the juice and the concentration of the acidic or basic constituent respectively. In the stomach as the rate of secretion increases the concentration of hydrochloric acid increases and approaches a limiting value, namely the concentration of acid in the pure parietal secretion.[6] Similarly, as the rate of pancreatic secretion increases the bicarbonate concentration increases and approaches a limiting value, which, as in the case of the acid of the stomach, is approximately equal to the osmolar concentration of blood plasma.[7]

This concept of participation in elaboration of secretion by the small duct epithelial cells is offered as an hypothesis; much more evidence is required to establish or disprove its validity.

(Scanlon, Catchpole and Gersh[8] have also found a decreased responsiveness to secretin in alloxan diabetic dogs).

5 Babkin, B. P., *Secretory Mechanism of the Digestive Glands,* P. B. Hoeber, New York, 1944.

6 Gray, J. S., *Gastroenterology,* 1945, **1**, 390.

7 Hart, W. M., and Thomas, J. E., *Gastroenterology,* 1945, **4**, 409.

8 Personal communication from J. H. Scanlon, H. R. Catchpole, and I. Gersh, Naval Medical Research Institute, Bethesda, Md.

15494

Exposure of Guinea Pigs to Intermittent High Oxygen Tension and Its Failure to Depress Erythropoiesis.*

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In recent years the theory has been advanced that the rate of erythropoiesis is regulated by the oxygen tension in the bone marrow. It is well known that exposure to low oxygen tension over a sufficient period of time will produce a marked degree of polycythemia. However, there appears to be little unanimity of opinion among investigators about the effects of high oxygen tensions in man and animals.

Barach and McAlpin[1] found no significant alterations in the erythrocyte counts of patients with polycythemia vera following continuous exposure to 50% oxygen for 15 to 17 days. Reinhard, Moore, Duback and Wade[2] found that subjecting patients with sickle cell anemia to a continuous supply of 70-100% oxygen for 8 to 20 days caused a significant decrease in reticulocytes and erythrocytes. Campbell[3] exposed mice, rats, guinea pigs, rabbits, monkeys, and cats continuously to 40-60% oxygen for 18 to 57 days at normal barometric pressure. In all but the cats he observed a decrease in the number of erythrocytes. In 2 guinea pigs exposed for 57 days there was an average decrease of 35% in the number of erythrocytes and 22% in the amount of hemoglobin. The reports of other investigators who have worked on this problem (Karsner;[4] Archard, Binet and Le Blanc;[5] Binet, Bochet and Bryskier;[6] and Davis[7]) are unconvincing.

The purpose of the present investigation was to determine whether prolonged *intermittent* exposure of guinea pigs to high oxygen tension would cause a depression of erythropoiesis. Two of us[8] have previously shown that intermittent exposure to low oxygen tension produces a marked stimulation of erythropoiesis.

Materials and Methods. Fourteen young adult male guinea pigs weighing 300 to 400 g were used in the first experiment. Four animals served as controls and the remaining 10 were exposed to 80-100% oxygen at normal pressure 6 hours a day 6 days a week for 8 weeks. For the administration of high concentrations of oxygen, a gas-tight cylindrical tank of approximately 16 cu. ft. capacity was used. The tank was just large enough to contain a cage equipped with food and water for the experimental animals. It had 2 glass windows through which the animals could be observed during the exposure, and was equipped with trays of soda lime to absorb carbon dioxide. A constant internal temperature was maintained by the circulation of cool water through a system of coils. Commercially pure oxygen (U.S.P.) was supplied by continuous flow to the tank, and the flow was regulated by a pressure reduction valve. To avoid building up a positive pressure a needle valve connected directly to the wall of the tank, but at the opposite end from the oxygen inflow valve, was used as a constant bleeder. Careful adjustment of this valve served to balance the amount of outgoing air with the incoming oxygen. During

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

1 Barach, A. L., and McAlpin, K. R., *Am. J. Med. Sc.*, 1933, **185**, 178.

2 Reinhard, E. H., Moore, C. V., Duback, R., and Wade, L. J., *J. Clin. Invest.*, 1944, **23**, 682.

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4 Karsner, H. T., *J. Exp. Med.*, 1916, **23**, 149.

5 Achard, G., Binet, L., and LeBlanc, A., *C. R. Acad. d. Sc.*, 1927, **184**, 771.

6 Binet, L., Bochet, M., and Bryskier, A., *J. de Physiol. et de Path. Gen.*, 1939, **37**, 524.

7 Davis, J. M., *J. Pharm. and Exp. Therap.*, 1943, **79**, 37.

8 Jensen, A. V., and Alt, H. L., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 384.

exposure, oxygen flowed into the tank at a rate of approximately 4.4 liters per minute.

After the experimental animals were sealed in the tank at the beginning of a daily exposure, the tank was flushed out with pure oxygen for 10 to 15 minutes, care being taken not to raise the tank pressure more than 10 mm Hg pressure over normal. The process of flushing required about 900 liters of oxygen. After flushing the oxygen concentration was determined. This initial concentration varied between 80 and 95% oxygen for all exposures. At the end of the 6-hour exposure period another oxygen concentration determination was made. This terminal concentration varied from 87 to 100% for all exposures. The average concentration of oxygen to which the animals were exposed was therefore about 92% which provided a partial pressure of oxygen of 699 mm Hg (4.4 times the normal). All oxygen concentration determinations were made by means of the Analox instrument.† Periodic observations of the experimental animals were made during exposures and at interval periods in an attempt to detect any abnormalities of behavior. The animals were weighed twice a week.

Blood studies were made on the experimental animals at the end of 183 hours exposure (covering 36 days) and at 287 hours (covering 57 days). The controls were studied at the same intervals. The animals were anesthetized with nembutal, and blood was drawn from the femoral artery 16 to 18 hours after periods of exposure to high oxygen tension. Erythrocyte counts were made with pipettes and hemocytometer chambers certified by the U. S. Bureau of Standards. The amount of hemoglobin was determined by the Duffie method[9] with an instrument calibrated by the oxygen capacity technic.

The second experiment was similar to the first except that the increase in partial pressure of oxygen was obtained principally by raising the barometric pressure in the tank. Six guinea pigs were subjected to 60 to 70% oxygen at 2 atmospheres pressure, which provided a partial pressure of oxygen of 998 mm Hg (6.3 times the normal). This was accomplished by first reducing the atmospheric pressure in the exposure chamber slowly to 400 mm Hg pressure below normal by means of an evacuation pump, and then gradually building up the pressure in the chamber with pure oxygen to one atmosphere over normal, and maintaining it there in the same manner as in the first experiment. The animals were exposed 6 hours a day 6 days a week, and received 17 exposures over a period of 20 days with a total exposure time of 106 hours. The number of erythrocytes and amount of hemoglobin were then determined.

Results. In the first experiment there was no significant change in the erythrocyte count and hemoglobin concentration after exposure to 80-100% oxygen at 1 atmosphere pressure. At the end of 183 hours of intermittent exposure covering a period of 36 days, the experimental animals showed a mean erythrocyte count of 5.29 (±.625) million per cu mm and a mean hemoglobin value of 12.9 (±.908) g per 100 cc. The control animals had a mean erythrocyte count of 5.11 (±.294) million per cu mm and a mean hemoglobin of 13.22 (±.511) g per 100 cc. The experiment continued with 9 experimental animals, one having died during the process of blood extraction presumably from an overdose of anesthetic. Blood was again taken from the animals at the end of 287 hours of intermittent exposure covering 57 days. The experimental animals then had a mean erythrocyte count of 5.22 (±.547) million per cu mm and a mean hemoglobin of 13.11 (±1.29) g per 100 cc. The controls had a mean erythrocyte count of 5.22 (±.646) million per cu mm and a mean hemoglobin value of 13.35 (±.983) g per 100 cc.

In the second experiment we also observed no change in erythropoiesis. After 106 hours of intermittent exposure to 50-60% oxygen tension at 2 atmospheres pressure the mean erythrocyte count for 6 animals was 4.81 (±.527) million per cu mm and the mean value for the amount of hemoglobin was 13.1 (±.538) g per 100 cc.

† Instrument produced by Oxygen Equipment and Service Co., Chicago, Ill.

[9] Duffie, D. H., *J. A. M. A.*, 1944, **126**, 95.

During both experiments the guinea pigs exhibited no adverse symptoms. They all ate readily, gained weight, and were normally active while in the tank and when they were put back into the storage cage with their controls during the interval period. No differences between the normal and the experimental animals could be observed.

Comment. Using the intermittent exposure method, it is apparent that high oxygen tension does not cause a depression of erythropoiesis in guinea pigs in contradistinction to the marked stimulation of erythropoiesis produced by low oxygen tension. With the continuous exposure method Campbell[3] observed significant reductions in the erythrocyte count and hemoglobin values in various species of animals. Because of the importance of this observation we exposed 6 guinea pigs continuously to 50-60% oxygen. Three animals died of pneumonia during the experiment. After 30 days, the remaining 3 animals had an average decrease of 21% in the erythrocytes and 13% in the amount of hemoglobin. Whereas these changes are not as great as those reported by Campbell, they further suggest that long continued exposure to oxygen may depress erythropoiesis in a normal animal.

The interesting experiment of Reinhard[2] *et al.* shows conclusively that erythropoiesis is depressed in patients with sickle cell anemia exposed to high oxygen tension. As such an effect was not obtained in patients with polycythemia vera (Barach and McAlpin)[1] it might be inferred that the hemopoietic equilibrium in sickle cell anemia is especially sensitive to increased oxygen pressures. The effect of prolonged high oxygen tension on the rate of erythropoiesis in animals and normal human beings warrants further investigation.

Summary. Guinea pigs were intermittently exposed to 80-100% oxygen at atmospheric pressure for 57 days, and to 60-70% oxygen at 2 atmospheres pressure for 20 days. The periods of exposure covered 6 hours a day 6 days a week. There was no significant diminution in the erythrocyte counts and hemoglobin values in any of these animals.

15495

The Effect of *Lithospermum* on the Mouse Estrous Cycle.*

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Introduction. Based on a report[1] that certain American Indians have employed the ingestion of the herb *Lithospermum ruderale* as a conception preventative, Cranston[2] performed a series of experiments on mice and found that a fluid extract of *Lithospermum* when mixed with normal diet would rapidly induce a suppression of the estrous cycles as well as a lowered birth incidence in breeding females. From inferential evidence Cranston concluded that the active factor in the herb operates directly on the pituitary gland, suppressing the formation or release of gonadotropic hormone.

Since this type of "anti-hormonal" action

* The work reported in this paper is a portion of that being carried on under a grant from the National Advisory Cancer Council. Thanks are hereby extended to Dr. H. B. Andervont of the National Cancer Institute for having supplied us with C_3H stock, and to Dr. S. H. Hutner of the Haskins Laboratories for many helpful suggestions relating to the problem under study.

1 Train, P., Hendricks, J. R., and Archer, W. A., *Medicinal Uses of Plants by Indian Tribes of Nevada*, Part II, p. 102. Issued by the Division of Plant Exploration and Introduction, Bureau of Plant Industry, U. S. Dept. of Agriculture, Washington, D.C., 1941.

2 Cranston, E. M., *J. Pharm. and Exp. Therap.*, 1945, **83**, 130.

is pharmacologically unique, work was undertaken (1) to confirm the effect of the drug on the estrous cycles of at least 2 unrelated strains of mice, and (2) to determine the effect of the drug on the incidence and development of mammary tumors, the appearance of which, in high tumor strains, is known to implicate the pituitary-ovarian endocrine system.

The latter study is still in progress. The former constitutes the subject of this note.

Experimental. Data were collected on 2 strains of mice: C_3H high mammary tumor females bred in this laboratory from stock obtained originally from the National Cancer Institute, and a heterozygous line of low-mammary-tumor albino mice.

Powdered *Lithospermum* from the whole plant, collected and dried in Montana, was fed in pellet form at a 15% level, the remaining 85% consisting of powdered Rockland Mouse Diet. The 15% level was chosen by extrapolation from a curve based on toxicity tests with groups of Rockland mice receiving 5%, 10%, 20%, 30% and 40% *Lithospermum* for a period of one month during which time a careful weight record was kept. The 15% level was the maximum dosage not affecting the weight of the fed animals.

Vaginal smears were made by the lavage method, with the use of distilled water and a fine pipette. The smears were dried under heat and stained with aqueous methylene blue. Differential counts were made under high dry magnification and approximately 5 fields were counted for each determination.

Twenty-four C_3H females 6-8 months of age were placed on the experimental diet and vaginal smears were taken daily for one month, after which smears were made weekly. Smears of 5 normal-diet females served as control. The animals were maintained on the diet for 3-5 months, during which time estrus occurred only rarely and in only a few of the mice. Generally speaking, the response in the C_3H strain to *Lithospermum* was immediate and persistent, in contrast to a type of refractoriness to the drug developed by some of the Rockland mice, described below.

Twenty Rockland females 2 months old were put on the 15% *Lithospermum* diet, with 10 females on normal diet serving as controls. Vaginal smears on the Rockland mice were taken daily for a period of 3 months. These smears were read differentially for percentage of cornified cells, nucleated epithelial cells and leucocytes. Four typical graphs of cornified cell levels are shown in Fig. 1. In contrast to the uniform response of the C_3H strain, the Rockland mice showed a great degree of individual variation, ranging from complete absence of response to full and continuous response. About 20% of the animals are in the former category, and 15% in the latter. The majority of the animals, however, (65%) went into an initial anestrus but came into estrus at about 30-day intervals with prolonged periods of anestrus between. Some of the mice at the end of several months developed a complete refractoriness to the drug, apparently recovering their normal cycles, though still on the *Lithospermum* diet, indicating either the development of an antagonist to the drug or an elevation of the organism's response threshold. Some evidence for the development of such a refractory state can be seen in 2 of Cranston's animals.[2]

Weights were taken on the Rockland animals at the conclusion of the experimental feeding. The control animals averaged 36.6 g and the *Lithospermum*-fed animals averaged 37.1 and 36.1 g, with no evidence of abnormal scatter. Cranston, using younger mice in experiments which involved administration of *Lithospermum* at rather higher dosage levels than employed by us, observed initial weight losses from which, however, the mice recovered while still on the diet.

The ovaries and uteri of females in *Lithospermum* anestrus showed marked atrophy, being about one-fourth normal size, with ovaries, especially in the C_3H strain, containing largely atretic follicles. Animals, however, which after long periods of anestrus became refractory to the drug and again developed estrus, had smaller than normal ovaries which, nevertheless, contained what appeared to be functional follicles.

Discussion. That a factor in *Lithospermum*

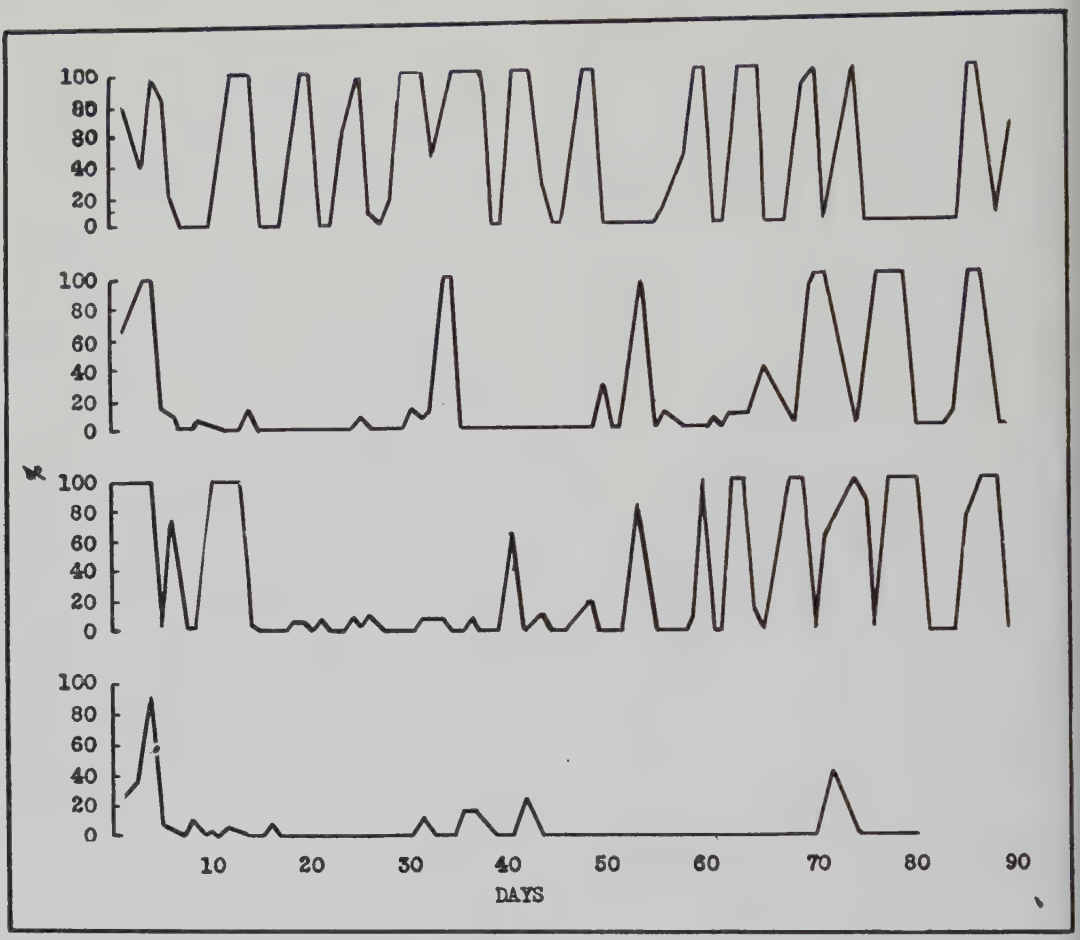

FIG. 1.

Graphs showing the percentage of vaginal cornified cells in female Rockland mice, over a period of about 90 days. Smears were made every 24 hours by the lavage method. The 4 sets of curves are: (top) of typical control mouse on standard diet; (two center) 2 examples of initial anestrus, followed by refractoriness in mice on a 15% *Lithospermum* diet; (bottom) example of persistent anestrus in mice on a 15% *Lithospermum* diet.

induces a suppression of estrous cycles in mice is confirmed. It is of interest to inquire into the nature of the mechanism of this action.

It is well known that a similar suppression of estrous cycles may result from a number of conditions, such as, inanition, deficiency of almost any of the vitamins, the action of injected "anti-hormone" substances, injected estrogens, androgens, progesterone, etc. It is doubtful, however, that *Lithospermum* operates through any of these media. General inanition may more or less be ruled out because of the fact that the *Lithospermum* effect can be achieved at dosages which do not affect body weight or general metabolic activity.[2] That a vitamin deficiency is involved is also doubtful. Vitamin A deficiency prevents the appearance of normal estrus and the condition is characterized by a persistent cornification of the vagina,[3] a symptom not present in animals in *Lithospermum* anestrus. Evans and Simpson[4] have produced permanent anestrus in rats by feed-

[3] Evans, H. M., and Bishop, K. S., *J. Metab. Research*, 1922, **1**, 335.

[4] Evans, H. M., and Simpson, M. E., *Anat. Rec.*, 1930, **45**, 215.

ing a Vitamin B deficient diet. This anestrous condition yielded to implants of normal pituitary tissue, indicating that such a deficiency results in decreased gonadotropic potency of the pituitary. The exact mechanism by which vitamin E deficiency produces sterility is still subject to controversy; however, there is no doubt but that its effects are irreversible. The fact that a refractoriness to *Lithospermum* may develop in mice would seem to negate any hypothesis proposing that *Lithospermum* activity is dependent upon the induction of a deficiency of vitamins B and/or E.

The effect of estrogens and androgens in interfering with gonadotropic action cannot be considered to be similar to the *Lithospermum* effect because the latter is entirely free of estrogenic or androgenic potency.

That the mechanism of *Lithospermum* activity involves the thyroid has been doubted by Cranston[2] who found no significant change in the weights of the thyroid glands or in basal metabolic rate after *Lithospermum* treatment. This view has been confirmed by us through histological examination of the thyroids of normal Rockland mice and those of mice on a ration of 30% *Lithospermum* for a period of 5 months. In both cases the alveolar epithelium was low to cuboidal with a normal amount of colloid storage, the experimental animals showing a slightly greater amount of interstitial tissue. The question may be raised as to whether such a histological picture might be expected if *Lithospermum* acts in the manner of some of the goiterogens of plant origin, specifically the seeds of the *Brassica* genus. Griesbach[5] has shown that a brassica seed diet induces hyperplasia of the thyroid follicles accompanied by typical goiterogenic changes in pituitary histology, *e.g.*, increase in the number of basophils with hyalinization and vacuolization, and a decrease in the number of acidophils. After 56 days, however, this effect becomes less pronounced at which time colloid is again stored in the thyroid follicles. This might indicate the appearance of a refractoriness to the brassica seed diet similar to the refractoriness developed to *Lithospermum*. Since the histological material examined by us was taken at a time when refractoriness had undoubtedly developed, it is impossible to state with certainty that the thyroid is not involved. However, the bulk of the evidence would seem to point against its primary involvement.

Cranston[2] by indirect experimental procedures has quite convincingly ruled out specific action of *Lithospermum* on the other endocrine glands except the pituitary, concluding that the action is directly on this gland, which in her experiments underwent marked weight loss during *Lithospermum* feeding and the resulting anestrus.

The fact that some mice, especially of the Rockland strain, develop a refractoriness to the herb action suggests either that the active factor is antigenic and that the organism builds up a protective antibody mechanism, or that the pituitary (if it, in fact, be the tissue affected) builds up a rising threshold of reactivity. That the refractoriness, however, may come and go in an almost cyclic manner (Fig. 1) suggests the existence of an obscure mechanism which with the data at present available cannot be illuminated.

The assumption that the *Lithospermum* action is via the pituitary gland is the basis for the further study now in progress of the effect of the drug on mammary tumor development. It is established that in high mammary tumor strains ovarian hormone activity is essential to the development of tumors. The milk factor apparently cannot operate without an adequate hormonal substrate. In suppressing the ovarian endocrine tissues by negating the pituitary gonadotropins, one might expect to break a link in the chain leading to mammary tumor development. Experiments are now in progress to determine whether such a rationalization is valid.

Summary. Female mice of C_3H strain when fed on a diet of 15% *Lithospermum ruderale* go into an immediate and persistent anestrus. Female mice of Rockland strain fed similarly also go into anestrus which,

5 Griesbach, W. E., *Brit. J. Exp. Path.*, 1943, **22**, 245.

however, may be followed by varying degrees of developed refractoriness to the drug, as evidenced in some mice by a reappearance of normal estrous cycles. The anestrous condition in both strains is accompanied by atrophy of the ovaries and uteri, and atresia of the follicles. Possible modes of action of the *Lithospermum* are discussed.

15496

Anesthesia. XXI. Anesthesia and the Steroid Hormones.*

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Selye[1-4] reported that the steroid hormones injected into certain species of laboratory animals produced anesthesia. In the generalization enunciated by Selye and referred to as "the fundamental law of steroid hormone anesthesia," this investigator states that "All compounds having a steroid-hormone action are capable of producing anesthesia while no compound devoid of hormone action possesses this power."[1] Indeed, Cashin and Moravek[5] had previously reported that they had anesthetized cats by injecting cholesterol suspensions intravenously. These experiments, in our opinion, appear to be inimical to the validity of Selye's generalization. In our studies on the effect of cholesterol on ether and pentothal sodium anesthesia, it appeared desirable to study further Selye's generalizations.

Experimental. Twenty-five rabbits received 2.5 cc/kg of 2% cholesterol suspension in a 25% solution of polyethylene glycol (Carbowax 1500) intravenously. Most of these animals appeared depressed. Six of them became unconscious and died promptly of acute pulmonary edema.

Fifteen dogs treated in a similar manner all manifested depression. Ten of these animals lost consciousness and died within 5 minutes.

Our attention was directed next to the hormonal steroids. Using the dosage schedules established by Selye we used the following compounds: progesterone, methyl testosterone, stigmasterol, estrone, benzestrol and diethylstilbestrol. Owing to the fact that in the "cat assay" of digitoxin, this glycoside in toxic quantities, produces a depression of the animal so that the anesthetic (ether) may be discontinued, we also used digitoxin in the series. In doses of 50 mg per rat weighing 92-205 g (average 158 g) not any of the foregoing compounds except progesterone and digitoxin produced "anesthesia."

Twenty-six animals, 8 male and 15 female, were used. None of the animals receiving digitoxin recovered from the comatose state produced by the drug. Three of the 9 animals receiving progesterone died.

Neither in the animals which died of progesterone nor with those that recovered was there ever a period in which the animal did not respond promptly to pain stimuli (pinching tail with hemostat). This syndrome cannot correctly be referred to as anesthesia when the sensory pathways are not blocked. In the rat, 6 mg/kg of

* The expense of this investigation was defrayed in part by a grant from the Ohio Chemical and Manufacturing Company of Cleveland, Ohio. The hormones used in these studies were furnished by Parke, Davis & Co.; Merck & Co., Inc.; Abbott Laboratories; Ciba Pharmaceutical Products; the Charles E. Frosst Co.; and Schieffelin & Co.

1 Selye, H., *J. Pharm. and Exp. Therap.*, 1941, **73**, 127.

2 Selye, H., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 116.

3 Selye, H., *Endocrinology*, 1942, **30**, 437.

4 Selye, H., *Anes. and Anal.*, 1943, **22**, 105.

5 Cashin, M. F., and Moravek, V., *Am. J. Phy.*, 1927, **82**, 294.

pentobarbital sodium intraperitoneally as well as 3 to 4 cc of ether by inhalation abolish response to these stimuli.

Conclusion. We question the suitability of the word anesthesia either in its restricted or in its extensional meaning as applied to the syndrome of cholesterol depression. Etymologically "anesthesia" means without sensation, but usage confines it to a description of a reversible process; unless accidentally it becomes terminal. We hold, therefore, that when this syndrome of depression produced by these steroid compounds is described as anesthetic, that the use of the adjective lacks preciseness, and that this loose choice of the word is misleading.

15497

Effect of Hypophysectomy During Early Proestrus on Ovulation in the Rat.*

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Long and Evans[1] have described the cyclic changes in the ovary of the adult rat and correlated them with the changes in the vaginal smear and, more recently, Boling *et al.*[2] correlated the changes in the ovary with estrous behavior. Smith,[3] Ascheim[4] and Zondek[5] have demonstrated the role of the anterior pituitary gland in maintaining ovarian function. Smith[6] described the profound atrophy of the gonads which invariably follows surgical removal of the anterior pituitary gland of the rat. Moreover, Fee and Parkes[7] showed that hypophysectomy in the rabbit will prevent ovulation, if the pituitary is removed within an hour after copulation; if more than an hour intervenes between copulation and hypophysectomy, ovulation will proceed normally. Since ovulation in the rabbit occurs approximately 12 hours postcoitum the data of Fee and Parkes suggest that the rabbit ovary has an intrinsic latency of about 11 hours in its ovulatory response to hypophyseal stimulation.

In a spontaneously ovulating form such as the rat the secretion of hypophyseal gonadotrophin (LH) may take place over a considerable portion of the estrous cycle before the concentration is great enough to produce ovulation. The present experiments were undertaken to determine whether, within a 2-hour period after the beginning of proestrus, there is a sufficient quantity of gonadotrophin in the blood to cause ovulation within the following 46 hours.

Materials and Methods. The regularity of the estrous cycles of a group of 70 young adult female rats of the Sprague-Dawley strain was determined by making daily vaginal smears during 2 or 3 cycles. On the day preceding the next calculated estrus the vaginal smears were made every 3 hours to determine more closely the onset of the proestrous phase. This procedure makes for a maximum error of 3 hours in determining the beginning of proestrus. As Long and Evans[1] have shown, the beginning of

* This work was supported by a grant from the Wisconsin Alumni Research Foundation.

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1 Long, J. A., and Evans, H. M., *Memoirs Univ. Calif.*, 1922, **6**, 1.

2 Boling, J. L., Blandau, R. J., Soderwall, A. L., and Young, W. C., *Anat. Rec.*, 1941, **79**, 313.

3 Smith, P. E., PROC. SOC. EXP. BIOL. AND MED., 1926, **24**, 131.

4 Ascheim, S., *Z. f. Geburtsh. u. Gynak.*, 1926, **90**, 387.

5 Zondek, B., *Z. f. Geburtsh. u. Gynak.*, 1926, **90**, 372.

6 Smith, P. E., *Am. J. Anat.*, 1930, **45**, 205.

7 Fee, A. R., and Parkes, A. S., *J. Physiol.*, 1929, **67**, 383.

TABLE I.

Animal No.	Operation	No. tubal ova	No. corpora lutea	No. preovulatory follicles
1	None	4	4	1
5	"	6*	6	2
6	"	4	4	0
7	"	5	5	0
8	"	4	5	0
9	"	5*	5	0
2	Sham hypophysect.	5	5	0
3	" "	6	7	0
4	" "	4	5	1
1	Hypophysect.	0	0	4
2	"	0	0	5
3	"	0	0	5
4	"	0	0	2
5	"	0	0	6
6	"	0	0	5
7	"	0	0	3
8	"	0	0	4
9	"	0	0	4

* Number of ova difficult to determine because of beginning fragmentation.

proestrus is marked by the appearance of large numbers of uniform nucleated epithelial cells in the vaginal smear, to the exclusion of all other cell types. We adhered closely to the latter specification and did not regard as proestrous animals any individuals showing any degree of cornification of the vaginal epithelium, as determined by the vaginal smear method. Thus, from a group of 70 animals observed over a period involving 3 to 7 cycles, 18 animals were obtained which were within 3 hours of the onset of proestrus at the time of selection for experimental use.

Of this group of 18 early proestrous animals, 9 were hypophysectomized by the parapharyngeal approach within 2 hours of the time of selection. Of the 9 remaining animals, 6 were retained as unoperated controls and 3 were submitted to a sham hypophysectomy which included trephining of the sphenoid bone and tearing the dura mater. All animals were autopsied 48 hours following the onset of proestrus. The completeness of the hypophysectomy was checked by careful examination of the sella under the binocular microscope. The oviducts of all animals were ligated with fine silk at the tubo-uterine junction and the ovaries and tubes were then fixed in Bouin's solution.

Serial sections of one ovary and oviduct from each of the operated and control animals were stained with hematoxylin and eosin and prepared for microscopic study. The sections were examined carefully for the presence of incompletely formed corpora lutea, preovulatory type of follicles and for tubal ova.

Results and Discussion. The data are summarized in Table I. The ovaries of the animals which had been hypophysectomized in early proestrus all failed to ovulate, but from 4 to 7 follicles in the preovulatory phase were present.

In sham-operated and unoperated control animals microscopic study revealed from 4 to 7 tubal ova, a comparable number of incompletely formed corpora lutea and only an occasional preovulatory follicle. In some cases it was difficult to determine the exact number of tubal ova since their beginning fragmentation made it difficult to make an accurate count. Thus, in these cases, there was a slight discrepancy between the number of tubal ova and the number of incompletely formed corpora lutea.

The size of the residual cavity in the incompletely developed corpora of the sham-operated and control animals varied considerably. In some animals there was only a narrow rim of lutein tissue formed about a large follicular antrum, and in others there

was almost complete obliteration of the follicular cavity by newly formed lutein tissue. This variation in the development of the corpora lutea at a fixed time following the onset of proestrus is in keeping with the variation in the time of ovulation of different follicles within a single ovary.

The data support the conclusion that a hypophyseal hormone is required for ovulation and luteinization in the rat. It seems clear that this hormone must be secreted in sufficient quantities subsequent to the onset of proestrus, to produce ovulation and luteinization in the intact rat, as hypophysectomy in the early proestrus effectively prevents ovulation.

The procedure and results described in this paper provide the basis for further studies of the factors concerned with ovulation and corpus luteum formation in the rat.

Summary. Hypophysectomy of adult female rats within 2 hours after the beginning of proestrus prevents ovulation from occurring within a 46-hour postoperative interval. Sham hypophysectomy does not prevent ovulation as shown by the presence of partially developed corpora lutea and tubal ova. It is concluded that a hypophyseal hormone (LH) is required for ovulation and luteinization in the rat and that the quantity of hormone needed to produce ovulation and luteinization is secreted subsequent to early proestrus.

15498

Further Studies on Galactose Paralysis in the Rat.

B. H. ERSHOFF.

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The occurrence of paralysis has been observed in rats fed galactose on certain purified rations.[1] The purpose of the present experiment was to determine the effects of B vitamins on the incidence and severity of the above condition.

Procedure and Results. Female rats of the Sprague-Dawley strain were raised to maturity on a stock ration and selected for the present experiment at approximately 3 months of age and an average weight of 160 g (range 145 to 178 g). Four experimental rations were employed: diet I consisting of glucose alone; diet II of 50% glucose and 50% galactose; diet III of 50% glucose, 50% galactose and the following synthetic vitamins per kg of ration: thiamine hydrochloride 200 mg, riboflavin 400 mg, pyridoxine hydrochloride 20 mg and calcium pantothenate 200 mg; and diet IV of 40% glucose, 50% galactose and 10% yeast* providing the same amounts of the B vitamins as were present in diet III. Animals were placed in individual metal cages with raised screen bottoms to prevent access to feces, and sufficient food was administered to assure *ad lib.* feeding. Feeding was continued for 75 days or until death, whichever occurred sooner; and food and water intake determined daily for all rats.

Results are summarized in Table I. All animals fed galactose-containing diets developed a typical "galactose paralysis" consisting in early stages of an irregular and wobbly gait and progressing to the point of complete disuse of the hind limbs. One hundred per cent of the rats were affected, with severe paralysis developing in general by the 35th day. No significant differences in time of onset or severity of paralysis were observed on the various rations employed. Subsequent to the 35th day a rapid improvement occurred in the paralytic condition of rats fed the yeast-containing ration (diet IV); animals regained use of their limbs and by the 50th day no evidence of paralysis was observed in any of the surviving rats of this group. Recovery of paralyzed rats did not occur on the other rations

[1] Ershoff, B. H., *Am. J. Physiol.*, 1946, **147**, 13.

* Hi-Ribo No. 24, Anheuser-Busch, Inc., St. Louis, Mo. Each gram contained the following vitamin potencies according to the manufacturer: thiamine, 2 mg; riboflavin, 4 mg; pyridoxine, 100-170 μg; pantothenic acid, 200-250 μg; and nicotinic acid, 400-500 μg.

TABLE I.
Summary of Experimental Data.

Group	No. of animals	Initial body wt (g)	Dead wt of decedents (g)	Survival (days)*	No. of rats paralyzed (%)	No. of rats recovered from paralysis (%)	Avg daily intake per rat during 1st mo. of exp.	
							food (g)	water (cc)
Diet I	8	159.2	76.4 (8)	41.2 ± 3.6	0	—	7.7	6.0
II	12	160.4	71.6 (12)	37.9 ± 4.7	100	0	12.8	38.5
III	6	160.8	72.2 (6)	47.3 ± 3.7	100	0	13.4	43.5
IV	8	159.7	88.2 (3)	67.8+†	100	87.5	15.8	59.7

The values in parentheses indicate the number of animals which died of which this is an average.

* Including standard error of the mean calculated as follows: $\sqrt{\epsilon d^2/n} \;/\; \sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

† The experiment was terminated on the 75th day of feeding at which time 5 of the 8 rats in this group were still alive.

employed. All animals fed galactose-containing rations developed mature bilateral cataracts from the 18th to 27th day of feeding, no significant differences being noted in time of appearance or the subsequent condition of the cataracts on the various diets employed. Paralysis and cataract were not observed in animals fed glucose alone (diet I). Food and water intake as well as length of survival was significantly greater on diet IV than on other rations tested.

Although yeast did not prevent the occurrence of paralysis, rats on diets containing this nutrient recovered rapidly from their paralytic condition in contrast to the continued paralysis in animals similarly treated but receiving their B vitamins in synthetic form. These results indicate (1) that the paralysis was reversible and (2) that some factor(s) in yeast other than thiamine, riboflavin, pyridoxine or pantothenic acid was responsible for the above effect.

Although paralysis may readily be demonstrated in rats fed galactose on certain purified rations,[1] this condition has not been reported for animals fed galactose on a more complete ration,[2,3] indicating that the purified diets employed were deficient in some factor necessary for the prevention of the paralytic condition. Attempts to prevent paralysis, however, with a single food supplement have been unsuccessful. Neither glucose, butter fat nor corn oil when fed in conjunction with galactose has prevented paralysis.[1] Unpublished work from this laboratory indicates that a diet of 50% casein† and 50% galactose was similarly ineffective. In addition neither thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, calcium pantothenate, salt mixture‡ nor yeast* when fed with glucose and galactose was effective in this regard. Paralysis was readily prevented, however, by a diet of galactose 50.5%, casein† 25.0%, yeast* 10.0%, corn oil 10.0% and salt mixture‡ 4.5% together with vitamins A, D and E. Male and female rats of the Sprague-Dawley strain have been maintained in our laboratory for 9 weeks on the above diet with no adverse effects except the appearance of cataract. Similar results have been reported by Boutwell *et al.*[2] for a 6-week period in rats fed synthetic diets also containing galactose as the sole carbohydrate. Inasmuch as paralyzed rats in the

2 Boutwell, R. K., Geyer, R. P., Elvehjem, C. A., and Hart, E. B., *Arch. Biochem.*, 1945, **7**, 143.

3 Mitchell, H. S., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 971.

† Vitamin Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

‡ Salt Mixture No. 1, Sure, B., *J. Nutrition*, 1941, **22**, 499. Paralysis was not prevented by diets containing 45% glucose, 50% galactose, and 5% of salt mixture.

present experiment recovered on diet IV in the absence of fat soluble vitamins, it would appear that these substances were not responsible for the protective effect of the above diets. The data suggests, therefore, either that "galactose paralysis" is due to a multiple deficiency developing when the various supplements were fed singly or that rations indicated above permitted the synthesis either by the animal's own tissues or its intestinal flora of substances capable of preventing paralysis.

Summary. Rats fed a diet consisting of glucose and galactose developed a severe flaccid paralysis of the hind limbs. Addition of yeast or synthetic B vitamins to the above diet failed to prevent the appearance of paralysis, although recovery subsequently occurred in animals receiving yeast.

15499

An Improved Rabbit Holder.

Robert E. Shipley.

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In studies requiring repeated injections into the ear veins of rabbits, accidental extravasation with subsequent loss of the vein occurs not infrequently even under the best conditions. With the use of the conventional box-like rabbit holder there is rarely sufficient immobilization of the rabbit's head and ears to assure careful entry of the needle into the vein or to prevent its dislodgement by a sudden jerk of the animal's head.

Preliminary trials with a plaster mask shaped to accommodate the rabbit's head

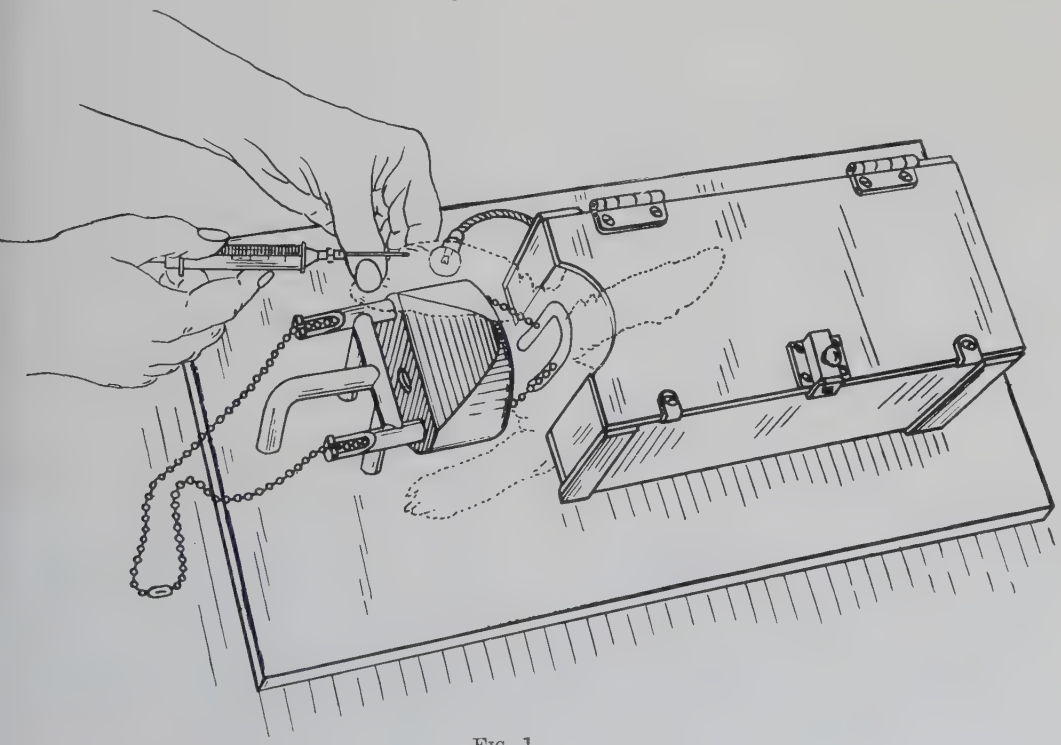

Fig. 1.

demonstrated that when the latter was rigidly supported the ears could be gently held and injected without mishap. After progressing through several models, a rabbit holder was designed according to the arrangement shown in Fig. 1, which aimed primarily at immobilization of the rabbit's head.

The head holder is made by molding a liquid thermo setting plastic* around a plaster of Paris model which has been shaped to conform to that of the head of a rabbit weighing about 2.5 kg. Provisions are made for leaving a hole from the nose to the outside. Within the 2 horizontal, tubular supports which are included in the plastic casting run two 3/16″ ball chains 12 inches long. The anterior three-fourths of the rabbit's head is held in the cast by a horse-collar-shaped neckpiece to which are attached the ball chains. Once the rabbit's head is placed in the cast, the operator exerts traction on the chains, and under moderate tension the latter are secured by fitting them into 2 narrow vertical slots on the ends of the horizontal support tubes.

The body of the rabbit is confined in a 5″ x 5″ x 12″ metal box with a hinged lid and clasp. Two flanges fastened to the front of the box prevent the rabbit's legs from reaching the area about the ears. All parts are mounted on a piece of 12″ x 27″ x ¾″ plywood, the top and edges of which are covered with sheet metal 0.050″ thick. For maximum rigidity, durability, and ease in cleaning, stainless steel, chrome-plated brass, and plastic were used. Wood and plaster of Paris are less durable but are otherwise adequate.

It has been found that by transilluminating the rabbit's ear from below, locating and visualizing particularly the smaller veins is greatly facilitated. With this procedure it is rarely necessary to clip or shave the hair over the vein in preparation for injection. A flexible arm with a small socket and bulb attached may be brought into any desired position beneath either ear.

The apparatus has been used several thousand times and has proved very satisfactory for rabbits weighing from 1.5 to 4 kg. Confining the rabbit in the holder causes no apparent discomfort.

Summary. A device is described which has been found to be of great advantage in effectively holding rabbits and immobilizing their ears for bleeding or injection procedures. The significant feature of the apparatus is a mask, shaped to accommodate the anterior three-fourths of the rabbit's head. An inverted, U-shaped collar holds the head firmly in the mask so that the position of the ears is correspondingly stabilized.

* "Catavar 101" (cream colored) or "Catavar 1001" (clear), Catalin Corporation of America, New York, N.Y. Plaster of Paris may also be used.

Acknowledgment is made to Mr. Clifford Wilson and to Mr. Roland Parker for suggestions in design.

15500

Retromammillary Inhibition of Cortically Induced Movement.

R. RHINES AND H. W. MAGOUN.

*From the Department of Anatomy, Northwestern University Medical School.**

Recent investigation has revealed 3 regions of the brain whose stimulation inhibits the response evoked from the motor cortex: cortical area 4-S,[1] the caudate nucleus,[2] and the bulbar reticular formation.[3]

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

1 Dusser de Barrenne, J. G., and McCulloch, W. S., *J. Neurophysiol.*, 1941, **4**, 311.

2 Mettler, F. A., Ades, H. W., Lipman, E., and Culler, E. A., *Arch. Neurol. Psychiat.*, 1939, **41**, 984.

3 Magoun, H. W., and Rhines, R., *J. Neurophysiol.*, 1946, **9**, 165.

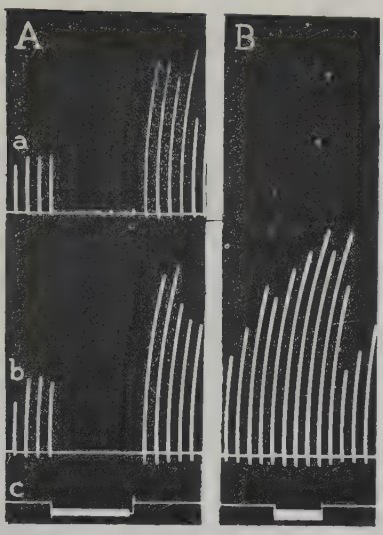

FIG. 1.
A. Cortically induced response of foreleg (a) and hindleg (b) inhibited during stimulation (c) of midbrain tegmentum. B. Patellar reflex (b) slightly augmented during stimulation (c) of same tegmental point.

During an experimental study of brain stem facilitation of cortically-induced movement,[4] it was observed that stimulation of a limited area of the rostral midbrain resulted in complete inhibition of the cortical motor response, while activation of a somewhat more extensive adjacent region produced depression of that response.

Methods. The left motor cortex of cats under light chlorolosane anesthesia was stimulated every 2 seconds by induction shocks and the responses of the right fore and hind legs were recorded with a kymograph. Using the Horsley-Clarke technic, the brain stem was stimulated in an exploratory fashion with 60 cycle sine wave current at intensities which did not by themselves evoke movement.

Stimulation of some of the points yielding inhibition was tested against the patellar reflex, elicited mechanically at 2-second intervals, and in one animal the midbrain tegmentum was stimulated against the flexor reflex of the foreleg, evoked every 2 seconds by exciting a cutaneous nerve.

[4] Rhines, R., and Magoun, H. W., *J. Neurophysiol.*, 1946, **9**, 219.

Results. The predominant alteration of cortically-induced movement during stimulation of the upper brain stem consisted of facilitation.[4] However, as shown in Fig. 1A, the response to cortical stimulation ceased or was diminished when the sites indicated in Fig. 2 were stimulated. Depression of response (small circles) was obtained from the hypothalamus and overlying thalamus, and complete inhibition (large circles) was elicited from the midline region of the posterior hypothalamus, dorsal to the mammillary bodies (Fig. 2A), from the retromammillary region (Fig. 2A and B) and from the tegmentum of the rostral midbrain (Fig. 2A and C). Stimulation of this region also inhibited responses evoked from the bulbar pyramid.

Stimulation of the same sites that caused inhibition of the cortical motor response, produced no change, or in a few instances slight facilitation of the patellar reflex (Fig. 1B). The flexor reflex was, however, inhibited by stimulating the same area of the midbrain tegmentum which inhibited cortically-induced movement, and inhibition of the flexor reflex was also obtained by exciting the periaqueductal grey.

Though these same areas were explored in the brain stem of the monkey, inhibitory responses were not encountered.

Discussion. If the sites whose stimulation depressed or inhibited cortically-induced movement in these experiments belong to a single neural system, it would appear to be distributed diffusely in the posterior diencephalon, to be concentrated ventromedially in the most anterior part of the midbrain and to shift dorsolaterally into the tegmentum at more caudal midbrain levels. The presence of intermixed facilitatory elements may, however, have prevented detection of a possible further distribution. The subsequent augmentation of cortical motor response after inhibition, seen in Fig. 1A, might, for example, have been produced by combined stimulation of excitatory and inhibitory components.

A rostral midbrain inhibitory influence is of interest from the point of view of 3 release phenomena which have been found to fol-

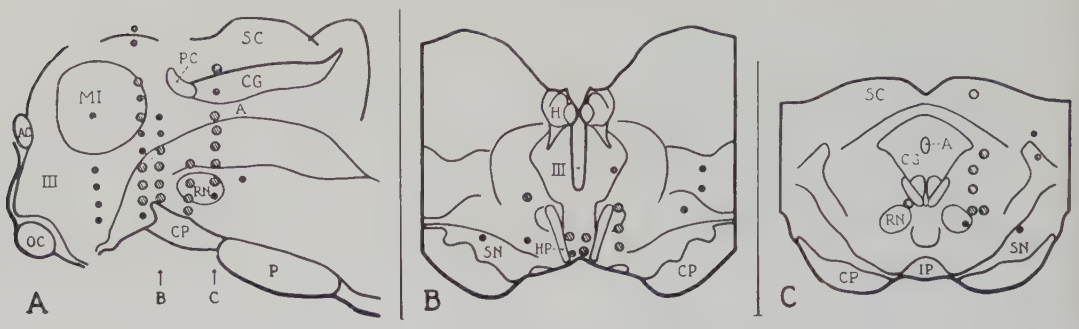

FIG. 2.

Distribution of sites whose stimulation completely inhibited (large circles) or depressed (small circles) cortically induced leg movement. A. Midsagittal reconstruction of cat's brain stem. B. Transverse section of retromammillary region. C. Transverse section of midbrain. Levels B and C are indicated by arrows in A.

A—Aqueduct
AC—Anterior commissure
CG—Central gray
CP—Cerebral peduncle
H—Habenula
HP—Habenulo-peduncular tract
IP—Interpeduncular nucleus
MI—Massa intermedia
OC—Optic chiasm
P—Pons
PC—Posterior Commissure
RN—Red nucleus
SN—Substantia nigra
III—Third ventricle

low injury to the mesencephalon. First, since Sherrington's discovery of decerebrate rigidity, it has been thought that one of the chief inhibitory centers from which lower brain stem levels are released in this condition is located in the midbrain. Ingram and his associates have made it clear that the red nucleus is not the exclusive midbrain inhibitory component eliminated by decerebration, as was once believed, though some rigidity does follow destruction of the red nuclei.[5] The sites yielding inhibition in the present study bear no obvious topographic relation to the red nucleus.

Second, a release phenomenon described as "obstinate progression" has been shown by Bailey and Davis[6] to follow destruction of the interpeduncular nucleus. Ventromedial midbrain sites whose stimulation yielded inhibition in the present experiments were located just rostral to the interpeduncular nucleus, but inhibitory responses were not obtained from this nucleus itself, nor from the habenulae or positions that were exclusively referable to the habenulopeduncular tract leading from it to the interpeduncular nucleus.

Third, ventromedial retromammillary lesions have been shown by Ingram, Barris and Ranson[7] to produce catalepsy in the cat. A large number of the inhibitory responses elicited in the present experiments were obtained from the area destroyed in such cataleptic animals, but to what extent injury to these inhibitory elements was involved in producing the cataleptic syndrome is at present uncertain.

Summary. A rostral midbrain area, the stimulation of which inhibits cortically-induced movement in the cat, has been described.

[5] Ingram, W. R., and Ranson, S. W., *Arch. Neurol. Psychiat.*, 1932, **28**, 483.

[6] Bailey, P., and Davis, E. W., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 307.

[7] Ingram, W. R., Barris, R. W., and Ranson, S. W., *Arch. Neurol. Psychiat.*, 1936, **35**, 1175.

15501

Effects of Ether and Curare on Neuromuscular Transmission.

WILLIAM SCHALLEK. (Introduced by Geo. H. Bishop.)

From the Laboratory of Neurophysiology, Washington University Medical School, St. Louis, Mo.

Anesthetists have noted that the amount of curare necessary to cause muscular relaxation is less in the presence of ether than of other anesthetics. Gross and Cullen[1] found that the contraction of the gastrocnemius muscle in response to electrical stimulation of its nerve is less in dogs anesthetized with ether than with other agents. They concluded that ether exerts a curariform action on neuromuscular transmission.

However these authors first determined the minimal strength of stimulus to which the muscle would respond, and then showed that after ether the muscle no longer responded to that strength of stimulus. Under these circumstances a failure of muscular contraction might be caused by a rise in the nerve threshold rather than by block of the neuromuscular junction. The present study avoids this difficulty by using maximal stimuli throughout. Each determination records the maximal response obtainable at that time.

In addition to the mechanical contraction, the electrical response of the muscle was also measured. This permits differentiation of the effect of ether on the contractile mechanism from its effect on the muscle action potential.

Method. The following studies were made on rats. The animals were anesthetized through a tracheal cannula attached to an ether bottle. The bottle was provided with a valve which controlled the amount of ether in the inspired air. Animals were kept lightly etherized throughout an experiment; when the effect of ether was to be studied the valve was turned to permit the maximal amount of ether to be drawn into the lungs. If natural respiration stopped, artificial respiration was applied by attaching an intermittent air pump to the side arm of the cannula. Natural breathing was usually resumed after a few seconds' artificial respiration.

In the experiments on curare Intocostrin (Squibb) was used. One cc of this preparation is described as containing the equivalent of 20 units of standard drug. It was injected into the femoral vein. The mechanical response was recorded through a lever attached to the tendon of the gastrocnemius, while the electrical responses were led off by needle electrodes inserted into the muscle. The height of potential was measured on an oscilloscope.

Results. The maximal etherization possible with the apparatus used blocks respiration before neuromuscular transmission. After 12 minutes respiration stops, while the electrical response of the muscle to maximal stimulation of its nerve shows an average depression of 12% (8 to 15) in 5 experiments. It is possible that the greater depression obtained by Gross and Cullen is due to their recording the mechanical contraction of the muscle. However a record of the mechanical response to maximal stimulation showed a depression of only 9% after 20 minutes of deep etherization. More probably the different results of Gross and Cullen are assignable to their use of minimal stimuli.

Since ether causes respiratory depression, the decreased muscular response might be due to anoxia rather than to direct action of ether. This possibility was tested by having the lightly etherized animal rebreathe into a balloon attached to the tracheal cannula. This kept the ether concentration reasonably constant, but permitted the oxygen concentration to drop rapidly. Two experiments showed an average depression of the muscle action current of 5% in 12 minutes. As a further check, after recovery the same animals were heavily etherized and oxygen was then blown in through the ether bottle. This increase in oxygen with little if any change in ether concentration caused an average recovery of 5% in 9 minutes. It appears

1 Gross, E. G., and Cullen, S. C., *J. Pharm. and Exp. Therap.*, 1923, **78**, 358.

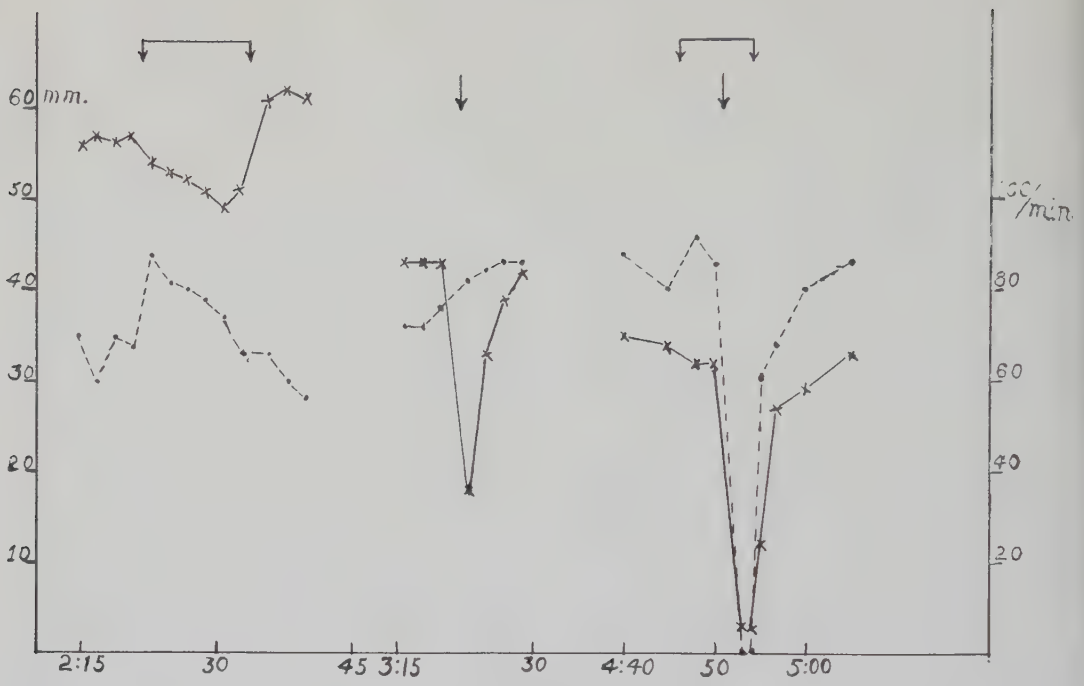

FIG. 1.

Effect of ether and curare on muscle action potential and respiratory rate in the rat. A progressive decline in the height of the action potential occurs during a long experiment, presumably due to deterioration of the muscle substance around the needle electrodes. Other experiments in which the order of the tests was varied show that this decline does not affect the conclusions. Intocostrin administered 0.1 unit per kg. Dash curves, rate of respiration, scale per minute at right margin. Full curves, height of muscle spike, scale at left margin in mm. Time on base line. Light ether throughout except between double arrows, deep ether. At single arrows, curare administered.

then that of the 12% depression following etherization, 5% may be caused by asphyxia.

The remaining depression is not enough to account for the observed increase of the action of curare in the presence of ether. That there is a synergism between the two agents is seen in Fig. 1. The maximal dose of ether alone caused a 14% depression of muscle action potential in this experiment, while 0.1 unit curare/kg caused an average depression of 46%, with minimal ether. Although the sum of the depressions caused by these agents given separately is 60%, the same dose of curare given 3 to 5 minutes after deep etherization has begun caused a depression of 91%. When the same dose of curare was given one minute after deep etherization began, the depression was only 72%, indicating that the ether had not yet reached the optimum concentration at the site of action.

In addition to this effect, a synergic action on respiration is shown by the same experiment. Ether alone stops respiration in 12 minutes, while this dose of curare used alone has no significant effect on respiration. But if this dose is given 3 to 5 minutes after deep etherization has begun, respiration stops 2 minutes after curare is injected.

Discussion. Although Gross and Cullen reported complete neuromuscular block under ether as tested by minimal stimuli, only a slight decrease of amplitude is found in the present study when maximal stimuli are used. This suggests that the depression which the above workers obtained may have been due to increase in the nerve threshold. The present paper does not permit localization of the peripheral action of ether. Since the depression obtained in the mechanical response was no greater than that obtained

in the electrical response, ether evidently acts proximal to the contractile process of the muscle. The synergism shown between ether and curare might suggest that they act at the same site, but this does not necessarily follow. Since we do not yet know the exact point at which curare acts, further speculation as to the peripheral action of ether does not seem warranted.

As noted above, curare has been reported to cause greater "relaxation" of muscles during anesthesia with ether than during the same degree of anesthesia with other agents. While this suggests that there is a greater synergism of curare with ether than with these other agents, another possibility exists. Anesthetists judge the degree of anesthesia to a large extent by the respiration, and amounts of ether and other agents which depress the respiratory center to the same degree may have different effects at other centers, as well as on the neuromuscular junction. A detailed examination of the actions of various anesthetics would be necessary to settle this point.

Summary. The response of the gastrocnemius to maximal stimulation through its nerve was studied in the rat. During deep etherization there is a depression of 12% in the muscle action potential. At the same time the respiration is depressed; anoxia alone due to this may account for 5% depression. Hence a muscular depression of only 7% can be attributed to the action of ether, not necessarily involving neuromuscular block. Nevertheless there is a synergism between ether and curare, since the effects of these agents given together is one-third greater than the sum of their separate effects.

Acknowledgment is due Dr. Geo. H. Bishop for advice in this research, and to Dr. S. M. Walker for assistance. E. R. Squibb & Sons furnished the intocostrin used.

15502

Effect of Enzyme Inhibitors on Transformation of Enzymes in the Living Cell.

JOHN M. REINER. (Introduced by M. B. Visscher.)

From the Department of Physiology, The Medical School, University of Minnesota.

Transformations in the enzymatic constitution of the living cell may occur as a result of gene mutation or as a physiological transformation in the presence of a constant genome. Changes of the latter type are most easily detected and studied in the cells of microorganisms, which are less specialized and more flexible than the cells of mature multicellular organisms, and present fewer complicating factors than the closely interrelated complex of cells found in the embryo. These changes have long been familiar to microbiologists; but they have until recently been the subject of much controversy, centering around the question whether they are only apparent transformations resulting from the action of natural selection upon pre-existing mutants in microbiological populations. Recent developments in the genetics of yeast[1] have made it possible to settle the controversy at least for this group of organisms; and it has been demonstrated by Spiegelman and coworkers[2,3] that modifications of enzyme constitution take place in genetically stable yeast strains, without the intervention of natural selection and without cell multiplication.

Most domestic strains of yeast readily ferment glucose, but do not ferment galactose. Some of them can, however, acquire the ability to ferment galactose after

1 Lindegren, C. C., *Bact. Rev.*, 1945, **9**, 111.

2 Spiegelman, S., Lindegren, C. C., and Hedgecock, L., *Proc. Nat. Acad. Sci.*, 1944, **30**, 13.

3 Spiegelman, S., and Lindegren, C. C., *Ann. Mo. Bot. Gard.*, 1944, **31**, 219.

TABLE I.
Effect of NaN_3 on Galactose Fermentation.

Time	Azide conc.:	Control	10^{-5} M	10^{-4} M	10^{-3} M
		Unadapted cells.			
90-180′	Microlitres	138	242	28	26
	% of control	—	175	20	19
		Adapted cells.			
10-120′	Microlitres	944			1314
	% of control	—			139

a period of exposure to this sugar under aerobic conditions. The time required for "adaptation" (a term introduced by Karström[4] to denote this process) is a characteristic constant for each strain of yeast. It is this enzyme transformation which has been most extensively studied by Spiegelman, and which is the subject of the present report.

The effect of several well-known enzyme inhibitors on the adaptation to galactose fermentation has been studied in the hope of casting further light on the nature of the process. In particular, we wished to obtain evidence bearing on the following crucial question: Is adaptation the synthesis of one or several new enzymes or coenzymes by the cell, or is it merely a lag within the metabolic cycle of the substrate itself, involving the formation of a certain level of intermediate products (*e.g.*, phosphorylated intermediates), as in the well-known induction period which occurs when glucose is fermented by dried yeast? Our results point strongly to the former alternative—the occurrence of synthesis.

Materials and Methods. A pure strain of *Saccharomyces carlsbergensis* (CLD-1A; obtained from Dr. S. Spiegelman) was grown in liquid culture containing peptone and 8% glucose, together with yeast extract and the necessary salts. For experiments, 48-hour cultures were harvested, washed several times with M/15 KH_2PO_4 to remove adherent medium, and finally suspended in enough M/15 KH_2PO_4 to give about 20 mg (wet weight) of yeast in 1 ml of suspension. Aliquots of 1 ml were distributed among conventional Warburg reaction vessels; enough galactose solution to make a final concentration of 4% was placed in the side-arm of each vessel; and enough distilled water, inhibitor or phosphate solution was added to the main chamber to bring the final volume of liquid to 2 ml. In occasional experiments the concentration of yeast or of galactose was varied, but in any one experiment the conditions in all vessels were strictly comparable. After equilibration on the Warburg bath, the substrate was tipped into the main chamber, the time at which this occurred being designated as zero time. Oxygen consumption and CO_2 production were followed at 15-minute or sometimes at 10-minute intervals by the usual 2-cup method.[5] The excess of CO_2 production over O_2 consumption in a given time period was taken as an index of fermentation and hence of adaptation rate. Inhibitor solutions were adjusted to a pH of 4.5 before use.

Experimental Results. The inhibitors chiefly used were sodium azide (NaN_3), sodium fluoride (NaF), 2-4-dinitrophenol (abbreviated DNP), and iodoacetic acid (abbreviated IAA). Their effects on unadapted and fully adapted cells were examined. Adapted cells were obtained by growing the yeast in the usual liquid medium with galactose substituted for glucose.

Azide is known as an inhibitor of hemin catalyses,[6] but also (unlike HCN and H_2S) as a specific inhibitor of syntheses even under anaerobic conditions.[7,8] This makes it particularly useful for our purpose.

The adaptation time of our yeast strain

[4] Karström, H., *Erg. Enzymforsch*, 1938, **7**, 350.

[5] Dixon, M., *Manometric Methods*, University Press, Cambridge, 1943.

[6] Keilin, D., *Proc. Roy. Soc. Lond.*, B, 1936, **121**, 165.

[7] Spiegelman, S., *Biol. Bull.*, 1945, **89**, 122.

[8] Winzler, R. J., *Science*, 1944, **99**, 327.

being 90 minutes, data obtained from unadapted cells are given only from 90 minutes on. With fully adapted cells, only the highest inhibitor concentration was used. Table I shows a typical set of results.

Despite the fact that the O_2 consumption (not shown) was just as completely inhibited by 10^{-3} M azide in adapted as in unadapted cells, the fermentation of galactose by adapted cells was not only not inhibited, but was actually stimulated by azide. None of the other compounds used gave such results; and we therefore present the data for them somewhat more briefly. Concentration curves were obtained, but we show here only the data obtained at the highest concentrations used. The stimulatory effect of azide at low concentrations will be discussed more fully elsewhere.

As in the case of azide, the effect of these compounds on O_2 consumption was very nearly the same for adapted and unadapted cells. Unlike azide, however, they inhibited the galactose fermentation equally well in both cases, with the exception of DNP. The results therefore leave open the question of the mechanism of inhibition.

TABLE II.
Effect of NaF, IAA, and DNP on Galactose Fermentation.
(Excess CO_2, % of control).

NaF 2x10-2 M	IAA 2x10-4 M	DNP 10-3 M
	Unadapted cells.	
0	14	3
	Adapted cells.	
6	0	40

We therefore attempted to determine whether the inhibition of unadapted cells could be reversed. Cells were exposed to the inhibitors for 90 minutes in the presence of galactose, then carefully washed free of adhering solutions, and tested for fermentation on fresh galactose. If any adaptation had occurred, the washed cells should have begun to ferment before 90 minutes. The results from 60 minutes onward are given.

With the possible exception of fluoride, the results appear negative. In the case of IAA, however, irreversible changes tend to occur after long periods of exposure. Fluoride[9] is known to combine with magnesium and phosphate as a complex salt within the cell, this complex then uniting with the enzyme enolase; and it is not certain that this complex would have been removed by washing the cells. Consequently, the results of Table III must be regarded with some reservations.

TABLE III.
Reversibility of Inhibition of Adaptation.
(Excess CO_2, % of control).

NaF 2x10-2 M	IAA 2x10-4 M	DNP 10-3 M
17	2	3

Discussion. The most clearcut results with inhibitors have been obtained with sodium azide. This poison prevents adaptation from occurring, but does not prevent galactose fermentation after adaptation has been allowed to take place. It does not interfere with the metabolic chain by which galactose is transformed into alcohol and CO_2. It must therefore prevent the formation of one or more essential enzymatic or coenzymatic links in that chain. This supposition is reinforced by the knowledge that azide does in fact inhibit synthetic processes, and does not inhibit normal glucose fermentation.

The poison DNP inhibits adaptation completely, but inhibits galactose fermentation by adapted cells to the extent of only 60%. It is known to have an effect on synthesis[10] even at rather low concentrations; and it may in these experiments be functioning in a mixed way—to inhibit synthesis of the adaptive enzyme or enzymes, and to block or slow up part of the process of fermentation.

The compounds NaF and IAA are known to block the Meyerhof fermentation scheme.[9,11] It is therefore likely that they merely prevent the adaptive enzymes from manifesting themselves, without necessarily preventing the adaptive enzymes from being formed.

9 Warburg, O., and Christian, W., *Bioch. Z.*, 1942, **310**, 384.

10 Clowes, G. H. A., and Krahl, M. E., *J. Gen. Physiol.*, 1943, **21**, 77.

11 Lundsgaard, E., *Bioch. Z.*, 1930, **217**, 162.

However, the data on this point are as yet not conclusive.

From the effects of azide in particular we conclude that adaptation to galactose fermentation involves the synthesis of one or more compounds which are not metabolic intermediates of galactose utilization, probably enzymes or coenzymes.

Summary. The effects of several enzyme inhibitors on the adaptation of yeast to galactose fermentation has been studied. The results strongly indicate that the process of adaptation involves the synthesis of one or more new enzymes.

The author wishes to thank Profs. M. B. Visscher and H. G. Wood for their support and encouragement of these studies.

15503

Influence of an Alkaline Solution on Tissue Toxicity of Uranium Nitrate.

WM. DEB. MACNIDER.

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The observation was made in this laboratory[1] that a solution of sodium carbonate would not only protect the kidney against the toxic action of a uranium salt but that it would furthermore protect it against the injurious effect of a general anesthetic body. The first part of this observation was confirmed by Goto[2] and very recently further extended by the studies of Donnelly and Holman[3] who were able to demonstrate a similar order of protection from a solution of sodium citrate. In a later paper[4] these authors and their associates attempt an explanation for this phenomenon of protection. They attribute it not to the action of a solution of sodium citrate as an alkaline medium but they believe that their "data on sodium citrate protection against uranium injury point to the maintenance of one or more vital equilibria (possibly the 'citric acid cycle' of carbohydrate metabolism) while the normal processes of repair are taking place, for we have no evidence that repair is accelerated or altered in any way." It becomes difficult to see how these authors could come to such a conclusion without employing control experiments in which sodium carbonate[1] or sodium bicarbonate[2] were used as agents to induce protection against uranium. Such an observation is especially appropriate when the fact is well established that citrates are rapidly and completely changed in the tissues to carbonates.

The way in which an alkaline solution protects the kidney, the liver and likely other tissues against a uranium injury is not known. It is furthermore not known how uranium induces its injury. Holman and Douglas[5] have shown they were able to recover from 31 to 88% of uranium nitrate from the urine of dogs during the first 24 hours of an intoxication by 5 mg of this substance per kg of weight. It is at this period that the renal injury commences and rapidly progresses. The suggestion is here repeated[1] that the cellular toxicity of uranium salts may be due to their ability to inhibit processes of intracellular oxidation and that such an inhibition is due to the characteristic of the uranium atom to be radioactive. Such an inhibition would lead to an intracellular accumulation of hydrogen ions with a change in the chemical environment in which various oxidation reduction systems exert their

1 MacNider, Wm. deB., *J. Exp. Med.*, 1916, **23**, 171.

2 Goto, Kingo, *J. Exp. Med.*, 1917, **25**, 693.

3 Donnelly, G. L., and Holman, Russell, *J. Pharm. and Exp. Therap.*, 1942, **75**, 11.

4 Donnelly, G. L., Ross, C. J., Meroney, W. H., and Holman, Russell L., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 75.

5 Holman, Russell L., and Douglas, William A., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 72.

influence. The fact that such a tissue disturbance does exist is shown by the observation that a reduction in the reserve alkali of the blood invariably occurs in the dog during the course of a uranium intoxication.[6] With this order of reasoning, the suggestion is made that the intravenous use of an alkaline solution: carbonate, bicarbonate or citrate, exerts its tissue protection not by the neutralization of hydrogen ions in the sense that they are as such responsible for the tissue damage, but by maintaining through such a binding or neutralizing effect an intracellular chemical environment of such an order of balance (acid-base) that various enzymatic systems can operate in an effective manner as life processes.

[6] MacNider Wm. deB., *J. Exp. Med.*, 1917, **26**, 1.

15504

Effect of Parabiosis on Experimental Uremia.*

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From the Department of Surgery, University of Chicago, Chicago, Ill.

The purpose of these experiments was to study the efficiency of peritoneal anastomosis in parabiotic rats, one member of the pair being uremic.

Putnam[1] demonstrated in cats that fluids in the peritoneal cavity came into almost complete osmotic equilibrium with the blood plasma. Ganter[2] first studied peritoneal lavage as a therapeutic measure, noting that the introduction of salt solution into the peritoneal cavity of dogs with bilateral ureteral ligation improved the resulting uremic symptoms. It has been demonstrated that peritoneal lavage can reduce the values of nonprotein nitrogenous constituents of the blood[3–6] and prolong the lives of uremic dogs[7,8] and patients.[9,10] Frank, Seligman and Fine[11] eliminated uremia in a patient with acute renal failure by peritoneal irrigation.

A number of investigators[12–15] have studied renal function by the method of parabiosis. Herrmansdorfer[12] joined pairs of rats in parabiosis with coelioanastomosis and later removed the kidneys of one of the parabionts; he was able to remove 3 kidneys from the 2 rats and maintain life without a rise in nonprotein nitrogen, or other constituents of the blood. All of these investigators produced

* This study was aided by a grant from Mr. Ben May, Mobile, Alabama, from the Albert and Mary Lasker Foundation, Inc., and from the Sidney and Frances Brody Foundation.

[1] Putnam, T. J., *Am. J. Physiol.*, 1923, **63**, 548.

[2] Ganter, G., *München. med. Wchnschr.*, 1923, **70**, 1478.

[3] Landsburg, M., and Groinski, H., *C. R. Soc. de biol.*, 1925, **93**, 787.

[4] Rosenak, S., and Siwon, P., *Mitt. a. d. Grenzgeb. d. Med. u. Chir.*, 1926, **39**, 391.

[5] Heusser, H., and Werder, H., *Bruns' Beitr. z. klin. Chir.*, 1927, **141**, 38.

[6] Curtis, G. M., and Pacheca, G. A., PROC. SOC. EXP. BIOL. AND MED., 1928, **26**, 874.

[7] Bliss, S., Kastler, A. D., and Nadler, S. B., PROC. SOC. EXP. BIOL. AND MED., 1932, **29**, 1078.

[8] Jeney, A. von, *Z. f. klin. Med.*, 1932, **122**, 294.

[9] Balaza, J., and Rosenak, S., *Wien. klin. Wchnschr.*, 1934, **47**, 851.

[10] Wear, J. W., Sisk, I. R., and Trinkle, A. J., *J. Urol.*, 1938, **39**, 53.

[11] Frank, H. A., Seligman, A. M., and Fine, J., *J. A. M. A.*, 1946, **130**, 703.

[12] Herrmansdorfer, A., *Deutsche Z. f. Chir.*, 1923, **178**, 289.

[13] Jeffers, W. A., Lindauer, M. A., Twaddle, P. H., and Wolferth, C. C., *Am. J. M. Sc.*, 1940, **199**, 815.

[14] Grollman, A., and Rule, C., *Am. J. Physiol.*, 1943, **138**, 587.

[15] Dannheisser, F., *Deutsche Z. f. Chir.*, 1931, **232**, 688.

the uremic state at a sufficiently long time after parabiosis for a common circulation to exist, while in the present experiments parabiotic union and uremia were established simultaneously. That an actual anastomosis of the 2 circulations eventually develops has been repeatedly shown by the passage of india ink, bacteria, avian blood cells, and dyes.[14,16–18] Studies made before the development of vascular communications are few; Spagnol[19] showed that colloidal dyes injected into one member of the pair did not cross to the mate during the first few days after anastomosis.

Experimental. Uremia was produced by bilateral nephrectomy in 8 adult albino rats and these rats died within 72 hours after operation. They became apathetic and later comatose; occasionally there was tetany. Fluid retention with massive edema and pleural fluid occurred. The nonprotein nitrogen of whole blood rose rapidly from the normal of 25 to 45 mg % to about 150 mg % in 24 hours, (Fig. 2) and to about 350 mg % before death.

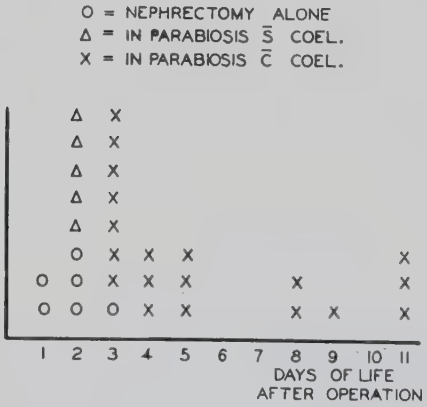

FIG. 1.

The duration of life in nephrectomized rats: after nephrectomy alone, 0; nephrectomy in a rat in parabiosis without peritoneal anastomosis, △; nephrectomy in one partner in parabiosis with coelio-anastomosis, X.

16 Hill, R. T., PROC. SOC. EXP. BIOL. AND MED., 1931, **28**, 592.

17 Hill, R. T., *J. Exp. Zool.*, 1932, **63**, 203.

18 Hill, R. T., PROC. SOC. EXP. BIOL. AND MED., 1932, **29**, 922.

19 Spagnol, G., *Arch. di fisiol.*, 1930, **28**, 157.

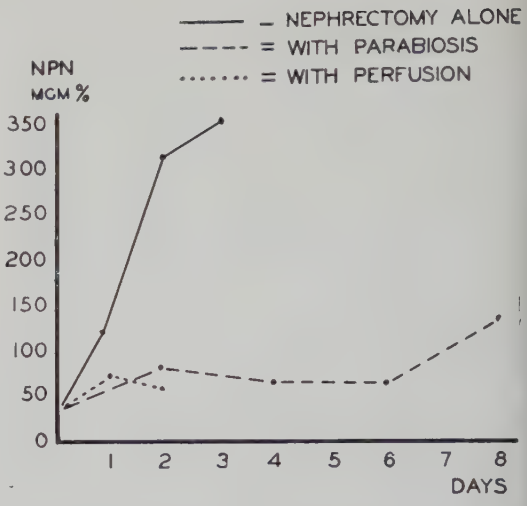

FIG. 2.

Nonprotein nitrogen of whole blood in nephrectomized rats.

The effect of parabiosis of such a nephrectomized rat with a normal rat was tried. A procedure similar to the original method of Sauerbruch and Heyde[20] was employed in which the skin of the animals was joined from neck to hip, the scapulæ were sutured together, and a large anastomosis produced between the 2 peritoneal cavities. The type of circulation obtained between the 2 animals was studied. Normal rats, injected intravenously with Evans' blue dye (T-1824) 3 cc of 1% solution, remain blue in color for many months. This amount of Evans' blue was injected intravenously in one rat, and during the first 4 days after parabiosis no transfer to the partner was observed as shown by the unchanged color of the animals and by colorimetric studies on the serum. After 4 days blue coloration was observed to extend into the skin of the uninjected rat for about 1 cm beyond the suture line. On the 6th to the 7th day the dye spread over the entire partner, and studies of the blood serum showed a nearly equal concentration of dye in the serum of both animals. It was therefore concluded that during the first 4 or 5 days the only form of communication was the common peritoneal cavity and the tissue spaces at the anastomosis.

20 Sauerbruch, F., and Heyde, M., *München. med. Wchnschr.*, 1908, **55**, 153.

Bilateral nephrectomy was performed on one of the rats of 22 pairs at the time of parabiosis. The pairs then lived for 3 to 11 days before the nephrectomized rat died. To insure that the coelio-anastomosis was of importance in maintaining life, parabiosis of a nephrectomized rat with a normal mate was done in 6 pairs without communication of the body cavities. All of these pairs died in 3 days or less, as did the single nephrectomized rats (Fig. 1).

Nonprotein nitrogen studies of whole blood showed that coelio-anastomosis was usually effective in maintaining low values, although shortly before death values of 100 to 200 mg % were sometimes reached. The nonprotein nitrogen of the nephrectomized rat usually remained between 75 and 100 mg % (Fig. 2) while that of the normal partner rose to 50 to 75 mg %.

The ability of the partner to excrete phenol red (PSP) injected into the nephrectomized rat was determined. Although there was negligible excretion in the urine when the rats were in parabiosis without coelio-anastomosis, good values were obtained with peritoneal anastomosis. After 5 mg of PSP were injected intramuscularly 0.1 to 0.2 mg was excreted by the partner in the first 6 hours. The values tended to increase when repeated after 5 days, probably due to the development of an anastomosis of the circulations.

In 15 experiments the peritoneal cavity of a single nephrectomized rat was continuously irrigated with Tyrode's solution. Although the life of the nephrectomized rat was not lengthened over that of the nephrectomized control the nonprotein nitrogen remained at values between 75 and 100 mg %. Absorption of fluid took place and death apparently was due to increased pleural fluid, and pulmonary edema.

Conclusion. These experiments demonstrated the efficiency of the peritoneal membrane as a mode of excretion in renal insufficiency. The effectiveness of the natural dialyzor provided by coelio-anastomosis suggests the feasibility of developing an artificial dialyzing mechanism.

15505 P

"Dietary Factor" in Necrotizing Arteritis in Dogs a Lipid Substance.*

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During the past several years arterial lesions affecting principally the large elastic arteries (aorta, pulmonary artery, endocardium of the left auricle, and coronary arteries) have been produced with regularity by controlling 2 factors, diet and renal insufficiency.[1] The arterial lesions have been described and illustrated in previous publications, and the evidence for a dietary factor has been reviewed in a recent publication.[1]

In this paper is presented a summary of the data obtained from systematically testing the ingredients of the "standard diet" which was being fed at the time these unanticipated lesions were first encountered.

Method. The methods have been detailed in a previous publication.[1] Briefly, these consist of feeding a specified diet for a specified period of time (usually 8 weeks or longer), then damaging the kidneys (usually with heavy metal injury), and examining the arterial system both grossly and histologically when the animals die or are sac-

* This work was aided by a grant from The John and Mary R. Markle Foundation. We are indebted to Distillation Products, Inc., for the crystalline vitamin A and vitamin D used in these studies.

1 Holman, R. L., *Am. J. Path.*, 1941, **17**, 359; *Am. J. Path.*, 1943, **19**, 977.

TABLE I.
Influence of Diet on Incidence of Arterial Lesions Following Kidney Damage.

Diet	Weeks*	No. with lesions	
		No. in group	% positive
Standard (6% C.L.O.)	8-22	26/30	86.7
Kennel	?	5/111	4.5
Kennel + C.L.O.	8-15	22/25	88.0
Standard (corn oil substituted for C.L.O.)	8-13	0/6†	0.0

* Weeks of dietary feeding before production of kidney damage.
† One dog in this group had extensive necrotizing arteriolitis.

rificed (usually days or weeks after the renal injury).

"Standard diet," the diet that was being fed at the time these unanticipated lesions were first encountered, consisted of calves' liver (raw wet weight), 32 parts; cane sugar, 25 parts; corn starch, 25 parts; butter, 12 parts; and commercial cod liver oil (USP XI—vitamin A, 850 I.U. per g, and vitamin D, 85 I.U. per g), 6 parts. Enough tomato juice was added to make a paste of which each gram contained 3 calories. The diet was fed in amounts to furnish 75 calories per kg per day. Five grams of kaolin and 1 g of salt mixture were added to each day's diet. Essentially, this is a low protein diet with only 7% of its caloric value derived from protein, 43% from fat, and 50% from carbohydrate. With the "alterations" in this diet, *i.e.*, doubling, halving, or omitting different ingredients of the diet, care was taken to keep it isocaloric.

"Kennel diet," unless otherwise specified, consisted of selected table scraps from the University dining halls.

Both of these diets were kept in a refrigerated room (38-40°F) and were fed in a reasonably fresh condition. In other words, there was no reason to suspect rancidity or other forms of spoilage.

Renal injury was usually produced by minimum lethal dosage of heavy metal which, in the case of uranium nitrate, consisted of 5.0 mg of uranyl nitrate per kg of body weight injected subcutaneously in the hypochondrium in 0.5% aqueous solution, and in the case of mercuric chloride, consisted of 3.0 mg per kg body weight injected intravenously in one of the external jugular veins in 0.1% aqueous solution.

Results. In this preliminary report the detailed data obtained from doubling and halving the various ingredients of the standard diet are omitted, and all of the pertinent data to date are combined in one simplified table (Table I). Suffice it to say that all the negative as well as all the positive data that have been obtained point to something of lipid nature that (in dogs at least) has to be fed for a period of 8 weeks or longer before experimentally-induced kidney damage is regularly followed by arterial lesions.

Analysis of the data in Table I yields 3 types of evidence: (1) definite evidence for a dietary factor; (2) evidence that the dietary factor is contained in commercial cod liver oil; and (3) evidence that the dietary factor is not unique to cod liver oil—the finding of typical arterial lesions in 5 control dogs, *i.e.* dogs fed only kennel diet for an indefinite period before their kidneys were damaged, all occurred during an 18-month period when kennel diet consisted of beef bones with much adherent fat;[2] and the dog fed corn oil instead of cod liver oil that had extensive necrotizing arteriolitis is additional evidence that the dietary factor is not unique to cod liver oil. All the data implicate a substance (or substances) of lipid nature. The dietary factor is heat stable, is not readily oxidized, is not vitamin A, and is not vitamin D. Studies designed to further identify the dietary factor are in progress.

Comment. There is one important difference between these studies and previous

[2] Holman, R. L., *J. Exp. Med.*, 1945, **81**, 399.

ones[3] on arterial lesions related to cod liver oil—*renal insufficiency*. The manner in which renal insufficiency is produced (heavy metal injury, bilateral nephrectomy,[4] *Leptospira canicola*[5] is relatively unimportant, but some degree of renal damage is *essential*. The "standard diet" or kennel diet plus cod liver oil can be fed to dogs indefinitely (at least as long as a year) and no arterial lesions are ever observed until renal function is disturbed. This emphasizes the role of the kidney in the internal metabolism of the "dietary factor," which presumably is lipid in nature.

As time goes by, we gain confidence that arterial lesions can be produced with regularity in dogs by controlling 2 factors, (1) diet and (2) kidney damage; that both factors are necessary, and that only these 2 factors are involved.

[3] Agduhr, E., and Senstrom, N., *The Appearance of the Electrocardiogram in Heart Lesions Produced by Cod Liver Oil Treatment*, Uppsala, Almguist and Wiksells, 1930; Cowdry, E. V., *Arteriosclerosis*, New York, The Macmillan Co., 1933.

[4] Holman, R. L., *Am. J. Path.*, 1943, **19**, 159.

[5] Holman, R. L., unpublished data.

15506

Pigment Studies on the Incisor Teeth of Vitamin E Deficient Rats of the Long-Evans Strain.*

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Several investigators[1-5] have reported that vitamin E deficient rats, over a period from 45 to 222 days, lose their natural brownish yellow pigment of the maxillary incisor teeth. During the past 9 years we have had occasion to examine several hundred vitamin E deficient rats of the Long-Evans strain. Although we did not check specifically for abnormal white incisors, we feel reasonably sure that such an obvious change in the pigmentation of the teeth would not have passed entirely unnoticed. To check accurately this depigmentation phenomenon in our strain of rats, the following experiment was conducted.

Procedure. Four groups (Groups 3, 5, 9, 11) of rats were maintained on our vitamin E deficient Diet No. 5† for either 310 or 460 days. Other groups (Groups 1, 7) were given Mason's vitamin E deficient Diet No. 69‡ for 130 days. Each of these groups was compared with normal control groups fed a commercial dog biscuit diet plus bi-

* Aided by a grant from the Milbank Memorial Fund of New York. The following materials were generously contributed: brewers' yeast by The Vitamin Food Company of Newark, N.J., and the cod liver oil by E. R. Squibb and Sons of New York City.

[1] Dam, H., and Granados, H., *Science*, 1945, **102**, 327.

[2] Davis, A. W., and Moore, T., *Nature*, 1941, **147**, 794.

[3] Granados, H., and Dam, H., *Science*, 1945, **101**, 250.

[4] Granados, H., and Dam, H., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 295.

[5] Moore, T., *Biochem. J.*, 1943, **37**, 112.

† Diet No. 5:

Casein (commercial)	24.0
Cornstarch (uncooked)	35.0
Salts No. 2	5.0
Lard	20.0
Cod Liver Oil	2.0
Brewers' Yeast	10.0
Cellulose Flour	4.0

All diet ingredients, except the cod liver oil, were mixed and then allowed to stand at room temperature for 2 weeks. The cod liver oil was added just before feeding.

TABLE I.

Maxillary Incisor Pigment Observations in Vitamin E-Deficient Rats.

(All rats were placed on the experimental diets at 21 days of age. The figures in the parenthesis represented the range of pigment values within each group. The unit 10 represented the maximum amount of normal pigmentation. Variations of the groups considered significant if P = 0.05 or less.)

Group	Diet	Days on diet	No. in group	Mean degree of pigmentation	S.E.	P.
		Females.				
1	E-Deficient No. 69	130	14	6.4 (6-9)	0.26	>0.05
2	Normal	130	12	7.0 (6-8)	0.23	
3	E-Deficient No. 5	310	18	7.3 (6-10)	0.13	<0.01
4	Normal	310	12	8.5 (6-10)	0.17	
5	E-Deficient No. 5	460	8	6.8 (6-8)	0.14	<0.01
6	Normal	460	7	8.4 (6-10)	0.27	
		Males				
7	E-Deficient No. 69	130	11	7.0 (5-8)	0.27	>0.05
8	Normal	130	13	7.6 (5-8)	0.21	
9	E-Deficient No. 5	310	15	7.5 (6-9)	0.11	>0.1
10	Normal	310	10	7.8 (5-10)	0.19	
11	E-Deficient No. 5	460	4	7.2 (5-9)	0.27	>0.1
12	Normal	460	5	7.6 (6-8)	0.14	

weekly supplements of fresh lettuce. During the last 60 days of the experiment the amount of pigmentation was carefully estimated with the use of a color chart with values 1 to 10. The value 10 representing an incisor tooth with a dark brown pigmentation, and the value 1 representing a chalky white incisor devoid of any pigment.

Results. The male rats in all groups at the termination of the experiment had almost identically the same degree of pigmentation in the maxillary incisors regardless of the diet used. The female rats, however, did show some slight variation. In the deficient animals the upper incisors were slightly less pigmented than in the control group. The largest variation was found in Groups 3 and 5 (Table I). The maxillary incisors of the control group had an average reading of 8.5, 8.4 as compared to 7.3, 6.8 of the deficient animals respectively. This is a significant difference but does not remotely approach the almost complete depigmentation in E deficient albino rats held on the vitamin E free diet for only 45 days as reported by Granados and Dam.[3]

The mandibular incisors in the deficient animals of both sexes uniformly had slightly higher pigmentation readings than the control rats. The greatest difference was in the 310-day-old male animals (Groups 9, 10). The deficient male group had a reading of 3.4 while the male control group had only a 2.3 reading. These were not sig-

‡ Mason's Diet No. 69:

Casein (commercial)	20.0
Cornstarch (uncooked)	50.0
Salts (Hubbell's)	2.5
Lard	18.0
Brewers' Yeast	7.5
Cod Liver Oil	2.0

nificant differences.

Discussion. It was obvious that our strain of rats was not showing the striking depigmentation phenomenon reported by other investigators. Several factors were investigated for an explanation for our findings. At the onset of this experiment we felt that the type and amount of iron used in the different diets might account for the discrepancies, since ferric iron is the cause of the pigment color.[1,4,5] Our Diet No. 5 contained 155 mg of ferric alum citrate per 100 g of diet. Mason's Diet No. 69 contained 50 mg of ferric phosphate per 100 g of diet. Since the rats on the latter diet (Groups 1, 7) failed to develop any significant depigmentation, we concluded that neither the differences in the iron compounds nor the unequal amounts of iron consumed by the rats were the determining factors in the failure of our rats to manifest any significant depigmentation of their incisors.

The presence of highly unsaturated fatty acids in the diet is a predisposing factor for depigmentation.[1] Both of the deficient diets, however, had equal amounts of cod liver oil and only slight differences in lard content (18-20%). Diet No. 5, however, had a shelf age of 2 weeks and was, therefore, slightly rancid upon feeding. In contrast, Diet No. 69 contained only fresh lard as it was prepared weekly and fed immediately to the animals. The fluctuation in the amounts of unsaturated fatty acids due to the incipient rancidity in Diet No. 5 is probably negligible. We believe, therefore, that the failure of our vitamin E deficient rats to show marked depigmentation of the maxillary incisors is not due to any obvious diet differences. Age and sex differences in our animals and those of other workers were not determining factors since our observations were on both sexes and the duration of our experiment equaled or exceeded that of the previous investigators.

The factor responsible for this disagreement with other workers on this problem is probably due to the difference in strain of our animals. All investigators, except one, reporting this peculiar depigmentation used albino rats. (Moore[5] used both albino and piebald rats). Our rats were inbred hybrids. The strain was started by Dr. Long in 1908 from a wild male grey rat and 2 tame albino rats.[6] The inbreeding of this hybrid strain may be the determining factor as it seems to be the only important variable not already accounted for. While no data are available on the effect of vitamin E deficiency on the wild grey rat, a recent note from Granados, *et al.*,[7] claimed that Syrian hamsters showed a greatly delayed depigmentation pattern as compared to the albino rat. They also reported that the Florida cotton rat showed no loss of incisor pigment after 150-180 days on an E-deficient diet. Thus this latter "wild" rat reacted to the deficiency in much the same manner as did our hybrid "wild" strain rats. It might be possible that these "wild" strains of rodents have the capacity to either manufacture or retain greater amounts of this tooth pigment than do the long established domesticated strains. Furthermore, Irving[8] also reported no depigmentation in the incisors of 3 female rats after 167 days on an E-deficient diet. Unfortunately, however, he did not indicate the strain of rat used.

Conclusions. 1. Vitamin E deficient rats of the Long-Evans strain did not show the extensive depigmentation of the maxillary incisors as reported by the other investigators. 2. Female rats maintained on an E-deficient diet for 310 days or longer gradually developed a slight incisor depigmentation that was statistically significant. Male rats on similar diets failed to manifest a significant change. 3. A comparison of diet differences, sex and age variations in our animals and those of other workers failed to provide an adequate explanation for our contradictory results. 4. It is suggested that a genetic difference between the Long-Evans and albino strains of rats may be the explanation for the failure of the former strain to show the maxillary incisor depigmentation earlier reported in vitamin E deficient albino rats.

6 Long, J. A., personal communication.

7 Granados, H., Mason, K. E., and Dam, H. (Proc. 1946 Meeting International Association for Dental Research), *J. Dental Research,* in press.

8 Irving, J. T., *Nature,* 1942, **150**, 122.

15507

Gonadotrophic Hormone Secretion in Immature Hypophysectomized Parabiotic Rats.*

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Following gonadectomy of one parabiotic rat of adult pairs, the other partner develops constant vaginal estrus if it is a female.[1,2] Likewise constant estrus appears in the hypophysectomized partner of adult hypophysectomized parabiotic rats following gonadectomy of the other parabiont.[2–5] In order to gain information concerning the kind and relative amount of gonadotrophic hormone released into the blood following gonadectomy in immature rats, experiments were designed in which parabiotic rats of both sexes were hypophysectomized and at the same time their parabiotic mates were gonadectomized. The gonad response of the hypophysectomized partner was used as the end-point in determining the nature of the pituitary secretion.

Materials and Methods. Littermate rats weighing 70 g or more were united in parabiosis at 31 to 33 days of age. The operative technic was that described by Bunster and Meyer,[6] except that metal skin clips were used instead of silk sutures in closing the skin incisions. On the 6th day after the animals were united, the left partner was hypophysectomized and the right partner was gonadectomized. All operations were performed under sterile conditions and under ether anaesthesia.

The majority of the animals were autopsied on the 11th day following the hypophysectomy-gonadectomy operation. The remainder were killed between the 3rd and 53rd days following this operation. At autopsy the gonads and accessories were removed and weighed. The ovaries were examined by the use of transmitted light to determine the qualitative response and the sella turcica was examined under binoculars and checked with the operation notes for completeness of the hypophysectomy.

Results and Discussion. The data reported in Table I give the weight and qualitative response of the ovaries of the hypophysectomized female following gonadectomy of its female or male partner. It will be noted that within 3 days following gonadectomy sufficient gonadotrophic hormone appeared in

TABLE I.
Ovarian Response of Hypophysectomized-Castrated Parabiotic Rats.

Sex of pairs	No. days hypophysectomized	Hypophysectomized partner: Ovarian wt, mg*	Hypophysectomized partner: Qualitative response
♀ ♀	3	29	Follicles
	5	26	,,
	6	32	,,
	7	26	,,
	7	33	,,
	7	15	,,
	9	24	,,
	9	36	,,
	10	32	,,
	10	11	,,
	13	13	,,
	17	11	,,
♀ ♂	4	26	,,
	8	31	,,
	10	50	,,
	10	23	,,
	10	37	,,
	10	40	,,
	10	30	,,
	53	59	,,

* The average ovarian weight of single hypophysectomized control rats after being hypophysectomized for 10 days was 4.0 mg.

* This work was supported in part by a grant from the Wisconsin Alumni Research Foundation.

1 Hill, R. T., *J. Exp. Zool.*, 1932, **63**, 203.

2 Meyer, R. K., and Biddulph, C., *Am. J. Physiol.*, 1941, **134**, 141.

3 Witschi, E., and Levine, W. T., Proc. Soc. Exp. Biol. and Med., 1934, **32**, 101.

4 Möller-Christensen, E., *Acta Path. et Microbiol. Scand.* (Suppl.), 1935, **22**, 1.

5 DuShane, G. P., Levine, W. T., Pfeiffer, C. A., and Witschi, E., Proc. Soc. Exp. Biol. and Med., 1935, **33**, 339.

6 Bunster, E., and Meyer, R. K., *Anat. Rec.*, 1933, **57**, 339.

the bloodstream to give a substantial ovarian response in the hypophysectomized female. This indicates that the pituitary gland increases its output of gonadotrophic hormone very soon after the gonads are removed. Regardless of the sex of the gonadectomized animal follicles only were present in the ovaries of the hypophysectomized female, at least for a period of 53 days following gonad removal. From this evidence it follows that FSH was released from the anterior pituitary gland of the gonadectomized rat and that it passed via the circulation to the hypophysectomized parabiont where the ovaries were either maintained at their prehypophysectomy level or were stimulated to develop follicles and to increase in size.

The question arose as to whether or not the ovaries contained only follicles because FSH was the only gonadotrophic hormone released from the pituitary gland or whether this was due to a refractory or unresponsive state of the ovaries of the hypophysectomized partner to LH. That the former is more likely true is indicated by the presence of corpora lutea in the ovaries of the hypophysectomized partner of one pair (not included in Table I) in which a fragment of pituitary gland remained in the sella turcica at the time of autopsy, the supposition being that the LH producing the luteinization came from this pituitary fragment. That the ovaries of the hypophysectomized partner were not refractory to LH is indicated by the results of experiments reported previously in adult hypophysectomized pairs[2] and in the accompanying paper,[7] which show that LH injected into a normal or a gonadectomized parabiont produces luteinization in the ovaries of its hypophysectomized mate.

From the above evidence it appears that LH is not released from the pituitary gland of a gonadectomized parabiotic rat during the early stages of castration, or if it is released, the concentration does not reach a sufficiently high level in the nongonadectomized partner to produce luteinization of the stimulated follicles of that rat. It would seem then that the luteinization of the ovaries of the intact parabiont which follows gonadectomy of the other rat, which has been reported in previous papers from this laboratory[8-11] and by others, is produced by LH from the pituitary gland of the intact rat. Evidently the stimulus effecting release of LH is the estrogen produced by the large stimulated ovaries of the intact rat, the FSH originating from the pituitary gland of the gonadectomized rat. However, it should be noted that the injection of estrogen into adult parabiotic rats does not cause the release of LH from the castrate pituitary gland.[2]

Hellbaum and Greep[12] have reported that the pituitary gland of normal adult female rats contains relatively large amounts of LH, and that of normal males is relatively deficient in this factor. Following castration the presence of LH in the pituitary of male rats becomes definitely evident about 20 days after gonadectomy as judged by assay of their pituitary glands. One of our female-male pairs was allowed to live for 53 days, but during this time only follicular stimulation was evident in the ovaries, which indicates that LH was not released by the pituitary gland, or if it was released the concentration did not reach a high enough level to produce luteinization.

Table II presents the results obtained when the hypophysectomized and gonadectomized parabionts were both males. It will be observed that there is considerable variation among the pairs in the weight of the testes, seminal vesicles and prostate of the hypophysectomized partner. Comparison of average weights of these structures with the corresponding average weights of normal animals indicates that there was some decline

7 Meyer, R. K., Biddulph, C., and McShan, W. H., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 95.

8 Meyer, R. K., and Hertz, R., *Am. J. Physiol.*, 1937, **120**, 232.

9 Hertz, R., and Meyer, R. K., *Endocrinology*, 1937, **21**, 756.

10 Biddulph, C., Meyer, R. K., and Gumbreck, L. G., *Endocrinology*, 1940, **26**, 280.

11 Biddulph, C., Meyer, R. K., and Gumbreck, L. G., *J. Exp. Zool.*, 1941, **88**, 17.

12 Hellbaum, A. A., and Greep, R. O., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 902.

TABLE II.
Weights Obtained in Hypophysectomized-Castrated Male Parabiotic Rats.

Sex of pairs	No. days hypophysectomized	Hypophysectomized partner* (mg)				Castrated partner (mg)		
		Testes	Sem. Vesic.	Prost.	Adren.†	Sem. Vesic.	Prost.	Adren.
♂ ♂	10	1733	130	177	17	10	22	28
	10	1760	92	122	12	8	8	30
	10	1212	20	51	19	7	17	39
	10	1438	162	172	20	12	26	25
	10	1516	129	144	24	9	27	26
	10	1900	251	166	15	12	19	30
	10	382	8	22	13	7	15	25
	10	1611	25	64	15	10	12	29
	10	1256	185	178	12	10	13	32
	10	1538	164	175	20	—	—	35
	10	1943	280	284	17	7	21	34
	10	1885	160	282	16	13	25	32
	10	1357	14	32	14	13	34	33
	11	645	13	22	16	8	14	39
	11	2141	375	313	12	9	16	25
	Averages	1474	133	147	16	9	18	31

* The testis, seminal vesicle and prostate weights of 7 normal animals of the same age were 2042, 101, and 170 mg, respectively

† The average adrenal weight of single hypophysectomized control rats of the same age was 10 mg.

in testis weight of the hypophysectomized partner, but that the accessory weights of this rat were approximately the same as those of normal controls. Usually when there was a marked decline in testis weight in a given rat the accessory gland weights likewise declined, which shows that the endocrine function of the testes decreases when the testes are not maintained by gonadotrophic hormones. It should be emphasized that in every case the testes of the hypophysectomized partner, regardless of their size and the size of the accessories, remained in the scrotum throughout the experiment. This is in agreement with the results of other workers.

The data obtained when the hypophysectomized partner was a female indicates that only FSH passed from the castrated male to the hypophysectomized partner. From the fact that neither the testes nor the accessories of the hypophysectomized male underwent the typical regressive changes of hypophysectomy, it is suggested that (1) endogenous FSH in the amounts present following gonad removal is concerned with the maintenance of the testes in the scrotum, for it seems logical to assume that FSH also passed from the gonadectomized male to the hypophysectomized male in these experiments, or (2) that small amounts of LH were also released from the pituitary gland following gonadectomy of the male and that these small amounts were sufficient to stimulate the interstitial cells of the testes to produce androgen, but were not enough to produce luteinization of the ovaries. The experiments reported in this paper on female-male pairs confirm the results of Greep[13] on parabiotic triplets which demonstrated that the gonadotrophins secreted following gonadectomy of a male produce only follicles in the ovary and stimulate the testes and accessory glands of the male.

Cutuly, *et al.*[14] have reported experiments with adult hypophysectomized-castrated male parabiotic rats in which varying degrees of gonadal and accessory maintenance in the hypophysectomized partner were obtained. Our results in immature animals are essentially the same as those reported by

[13] Greep, R. O., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 214.

[14] Cutuly, E., McCullagh, D. R., and Cutuly, E. C., *Endocrinology*, 1937, **21**, 241.

these workers in adult animals.

The variation in ovarian, testis and accessory weights in the hypophysectomized partner may be a reflection of the degree to which the pituitary gland undergoes castration changes following gonad removal. We have previously reported[11] that approximately 11% of a large number of control parabiotic rats failed to undergo the typical ovarian enlargement following gonadectomy of one parabiont. The percentage of rats failing to show the typical castration changes in this study when the recipient parabiont was hypophysectomized is slightly higher (17%), which indicates that the gonads of hypophysectomized rats respond less readily to gonadotrophic stimulation than those of normal rats.

Summary. Using immature hypophysectomized-gonadectomized parabiotic rats it has been demonstrated that following gonad removal in both sexes follicular stimulation only is obtained in the ovaries of the hypophysectomized parabiont for as long as 53 days following the operation. If the hypophysectomized partner is a male, the testes are maintained in the scrotum and their average weight is somewhat less than that of normals, whereas the weight of the accessory glands is approximately the same as that of normal animals.

Approximately 17% of the pairs failed to show any gonadal stimulation following the hypophysectomy-gonadectomy procedure. This percentage failure is slightly greater than that occurring in nonhypophysectomized pairs.

15508

Luteinization of the Ovaries of Immature Hypophysectomized Parabiotic Rats with Gonadotrophic Hormone Preparations.*

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It has been shown that following hypophysectomy of an immature female parabiotic rat and gonadectomy of its male or female partner only follicular stimulation is obtained in the ovaries of the hypophysectomized parabiont.[1,2] The question arose as to whether or not the ovaries of the hypophysectomized rat failed to become luteinized because they were unresponsive to luteinizing hormone (LH), or whether the condition of follicular stimulation was the result of a failure of LH to either be released from the castrate pituitary gland or to reach the ovaries in sufficient quantities to produce luteinization. In an effort to gain information on this question, one parabiont of a group of parabiotic rats was hypophysectomized and the intact rat was injected with gonadotrophic hormone preparations and the response determined in the ovaries of the hypophysectomized rat.

Materials and Methods. Littermate rats weighing 70 g or more were united in parabiosis at 31 to 33 days of age. The operative technic was that of Bunster and Meyer[3] except that metal skin clips were used instead of silk sutures in closing the skin incisions. On the 6th day following parabiotic union of the animals one partner was hypophysectomized and the other was either gonadectomized or left intact. All operations were performed under sterile conditions and under ether anesthesia.

Within a few hours after the operation,

* This work was supported in part by a grant from the Wisconsin Alumni Research Foundation.

1 Biddulph, C., and Meyer, R. K., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 92.

2 Greep, R. O., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 214.

3 Bunster, E., and Meyer, R. K., *Anat. Rec.*, 1933, **57**, 339.

TABLE I.
Injection of Gonadotrophic Extracts into Hypophysectomized Parabiotic Rats.

Condition of pair		Right partner			Left partner*	
Left	Right	Material injected	Days inj.	Sex of pairs	Ovarian wt, mg	Qual. response
Hypophysectomized	Gonadectomized	100 mg eq. per day LH 114	3	♀ ♂	24	Follicles
			5	♀ ♂	25	,,
			7	♀ ♂	39	,,
			7	♀ ♂	46	Foll. and C. L.
			10	♀ ♂	21	Follicles
			10	♀ ♂	37	,,
			10	♀ ♂	7	No. stim.
			10	♀ ♀	9	,, ,,
			10	♀ ♀	7	,, ,,
		100 mg eq. per day LH 54	10	♀ ♀	97	C. L.
			10	♀ ♀	64	,, ,,
			10	♀ ♀	77	,, ,,
			10	♀ ♀	48	,, ,,
	Normal	1.2 cc eq. per day pregnancy urine prep.	8	♀ ♀	48	,, ,,
			10	♀ ♀	40	,, ,,
			10	♀ ♀	46	,, ,,
			10	♀ ♂	50	,, ,,
	Gonadectomized	1.2 cc eq. per day pregnancy urine prep.	6	♀ ♂	112	,, ,,
			8	♀ ♂	133	,, ,,
			10	♀ ♂	185	,, ,,
			10	♀ ♂	179	,, ,,
			10	♀ ♀	117	,, ,,
			10	♀ ♀	103	,, ,,
			10	♀ ♀	153	,, ,,
	Normal	50 mg eq. per day unfract. sheep pit.	3	♀ ♂	41	Follicles
			3	♀ ♂	30	,,
			3	♀ ♂	30	,,
			3	♀ ♀	35	,,
			4	♀ ♀	30	,,
			7	♀ ♂	37	Foll. and C. L.
			10	♀ ♀	74	C. L.
			10	♀ ♀	78	,, ,,
			10	♀ ♂	99	,, ,,
			10	♀ ♂	55	,, ,,
			10	♀ ♂	14	,, ,,
			10	♀ ♂	49	,, ,,
			10	♀ ♂	60	,, ,,
		100 mg eq. per day unfract. sheep pit.	10	♀ ♀	157	,, ,,

* The average adrenal weight of the hypophysectomized partner was 17 mg; that of the other parabiont was 32 mg.

injection of either LH, gonadotrophin prepared from the urine of pregnant women, or unfractionated (FSH and LH)) sheep pituitary extracts was begun. Five-tenths cc of aqueous solution of the preparations was injected subcutaneously once daily into the gonadectomized or normal parabiont for 3 to 10 days following the hypophysectomy and gonadectomy. The various doses used are recorded in Table I.

At autopsy the ovaries of the hypophysectomized partner were removed and examined for their qualitative response and weighed. The adrenals of each partner were also dissected and weighed.

Results and Discussion. The results of the gonadotrophic hormone injections are found in Table I. It will be seen that both male and female rats were gonadectomized or left intact and injected with the various

preparations, the hypophysectomized partner always being a female. No difference is apparent in the results obtained with each preparation regardless of whether the gonadectomized or intact partner was a male or female.

The injection of LH54 produced an abundance of corpora lutea in each rat and the ovaries were heavier than those obtained with LH114. The latter preparation was prepared by a different method, and corpora lutea were found in the ovaries of only one animal, probably because of the low luteinizing activity of the preparation.

Injection of the pregnancy urine preparation into the normal rat of hypophysectomized-normal pairs produced corpora lutea in the ovaries of the hypophysectomized rat in all cases, the average ovarian weight being 41 mg. When the same preparation was injected into the gonadectomized partner of hypophysectomized-gonadectomized pairs it was found that corpora lutea were formed in the ovaries of the hypophysectomized rat in every instance, and the ovarian weight was much greater than before, the average being 140 mg.

The response obtained with the pregnancy urine preparation is an interesting one, for reports in the literature indicate that this type of gonadotrophin is by itself ineffective, or relatively so in hypophysectomized rats.[4-6] Regardless of whether the injected rat was normal or gonadectomized the pregnancy urine gonadotrophin used in this study luteinized the ovaries of the hypophysectomized rat.

The greater weight of the ovaries of the hypophysectomized-gonadectomized pairs was undoubtedly due to the greater stimulation of the ovaries by the combination of endogenous gonadotrophin from the gonadectomized rat's pituitary gland and injected gonadotrophin. It seems probable that the follicles present in the ovaries of the hypophysectomized partner of both types of pairs did not undergo involution immediately following hypophysectomy, and since the injections of the pregnancy urine preparation were begun immediately after the operation, the follicles became luteinized. In addition, the ovaries of the hypophysectomized partner of the hypophysectomized-gonadectomized pairs were apparently stimulated to produce new follicles by FSH from the pituitary gland of the gonadectomized partner.[1] The LH of the injected preparation acted on these stimulated follicles to produce luteinization, and consequently a greater ovarian weight was found in these pairs.

The injection of the unfractionated sheep pituitary preparation also produced luteinization of the ovaries of the hypophysectomized parabiont. In this group of animals the injections were made into the normal parabiont. A greater ovarian weight was obtained in the hypophysectomized rat with a high dose of the preparation than with a low dose.

Of interest is the fact that the adrenals of the hypophysectomized rat were partially maintained (weight 17 mg) by the adrenotrophic hormone from the nonhypophysectomized partner (adrenal weight 32 mg). The average adrenal weight of single hypophysectomized control rats of the same age was 10 mg.

From the above data it would seem that the presence of only follicular stimulation in the ovaries of the hypophysectomized-gonadectomized parabiotic rats in the previous study,[1] is due to the fact that the gonadotrophin secreted in the early stages of gonadectomy is largely FSH, little or no LH being secreted. Furthermore, the ovaries of the hypophysectomized parabiont are responsive to LH if sufficient quantities are present.

Summary. The injection of LH, gonadotrophin prepared from the urine of pregnant women, and unfractionated sheep pituitary extracts into the nonhypophysectomized partner produced luteinization in the ovaries of the hypophysectomized partner of either hy-

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5 Reichert, F. L., Pencharz, R. I., Simpson, M. E., Meyer, K., and Evans, H. M., *Am. J. Physiol.*, 1932, **100**, 157.

6 Evans, H. M., Meyer, K., and Simpson, M. E., *Am. J. Physiol.*, 1932, **100**, 141.

pophysectomized-gonadectomized or hypophysectomized-normal parabiotic rats.

The results indicate that the failure of the ovaries of the hypophysectomized partner of hypophysectomized-gonadectomized pairs to develop corpora lutea is due to an insufficient secretion of LH following gonadectomy rather than to an unresponsive state of the ovaries of the hypophysectomized animal.

15509 P

Feather Growth Rates in Thyroidectomized Hens Following Administration of Thyroxin.*

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The feather growth rates of normal fowl have been shown to be modified by the administration of thyroid[1,3] as well as by estrogens.[4-6] Although a general retardation in feather regeneration has been noted in spontaneous[7] and experimental[8,9] hypothyroid conditions in fowl, no measurements were previously made on feather growth in the various body areas. Consequently we were interested in studying the effects of thyroid removal on feather growth in brown Leghorn hens and to see if thyroxin administration had the same effect on growth rates in athyroidic hens as follows its administration in normal individuals.

For the study of feather growth rates, 25 papillae in 5 rows of 5 papillae each were selected and marked in comparable areas in each bird for each of the regions measured. Measurements were made at 2- or 4-day intervals on posterior breast, anterior breast, back and saddle. All individuals studied were adults and beyond 6 months of age.

Regenerating feathers of adult thyroidectomized females, operated on or before 10 days of age, did not become measurable until 12 to 14 days after plucking as compared to 9 or 10 days in normals. During the period of rapid growth, the breast and back feathers grew more rapidly than the saddle, as seen in their growth rates and lengths on the 48th day (Table I). This is in marked contrast to the order of growth rates in normal hens where posterior breast and saddle feathers grow more rapidly than back and anterior breast. Feather growth continued for a longer period in thyroidectomized hens than in normals. Breast and back feathers revealed a lesser reduction in length and growth rate than the saddle.

In view of the well-known feather growth-promoting action of thyroid preparations in normal fowl, it seemed advisable to study the effects of various doses of thyroxin on the slow-growing feathers of athyroidic hens.

Injection of 0.5 mg of thyroxin was followed by an increase in feather growth rates within 24 hours which persisted for 12 to 16 days (Table II). The maximum growth was attained between the 2nd and 4th days after injection when growth rates attained their normal level. With this as well as higher

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1 Torrey, H. G., and Horning, B., *Biol. Bull.*, 1925, **49**, 275.

2 Domm, L. V., *Anat. Rec.*, 1929, **44** (Suppl.), 227.

3 Juhn, M., and Barnes, B. O., *Am. J. Physiol.*, 1931, **98**, 463.

4 Domm, L. V., *J. Exp. Zool.*, 1927, **48**, 31.

5 Domm, L. V., and Gustavson, R. G., *Anat. Rec.*, 1929, **44** (Suppl.), 228.

6 Juhn, M., Faulkner, G. H., and Gustavson, R. G., *J. Exp. Zool.*, 1931, **58**, 69.

7 Landauer, W., *Am. J. Anat.*, 1929, **43**, 1.

8 Parhon, C. F., *C. R. Soc. Biol.*, 1924, **91**, 765.

9 Greenwood, A. W., and Blyth, J. S. S., *Proc. Roy. Soc. Edin.*, 1929, 49, 313.

TABLE I.
Feather Growth Measurements (in mm).

Ftr. age days	2 normal females				6 complete thyroidectomized females			
	Post. br.	Ant. br.	Back	Sad.	Post. br.	Ant. br.	Back	Sad.
				Average length.				
12	5.3	5.4	6.5	6.3	<1.0	<1.0	<1.0	<1.0
24	32.4	29.1	33.3	31.6	9.8	8.3	8.5	7.8
36	57.5	55.5	57.9	57.1	22.2	20.4	21.3	17.5
48	77.1	69.0	73.9	77.3	33.9	30.3	32.7	26.1
60	87.2	71.7	77.3	87.0	45.0	38.0	42.5	33.1
72	89.9	71.7	77.5	87.9	52.7	41.2	50.6	36.7
80					54.9	42.1	53.9	37.5
				2-day growth increments.				
12-24	4.5	3.9	4.5	4.2	1.6	1.4	1.4	1.3
24-36	4.2	4.4	4.1	4.2	2.1	2.0	2.1	1.6
36-48	3.3	2.2	2.7	3.4	1.9	1.7	1.9	1.4
48-60	1.7	0.4	0.6	1.6	1.8	1.3	1.6	1.2

TABLE II.
Modifications in Feather Growth Rates After Thyroxin Administration in Thyroidectomized Hens.

Dose, mg	Days inj. A	Ftr. areas	Two-day growth rate increments in millimeters Days measured post injection			
0.5	42		R-6-0	0-4	2-4	0-12
			A-36-42	42-48	44-46	42-54
		Ant. br.	1 7	3.7	4.4	3.0
		Back	1.6	3.0	3.8	3.0
		Sad.	1.7	3.1	3.7	3.1
1.0	19,		R-6-0	0-6	6-14	14-20
	26,		A-13-19	19-25	25-33	33-39
	33	Post. br.	2.3	3 8	3.9	4.9
		Ant. br.	2.2	2.7	5.2	5.1
		Back	1.9	3.6	4.0	3.8
		Sad.	1.0	2.5	4.0	4 6
1.5	18,		R-4-0	0-6	6-12	12-18
	24,		A-14-18	18-24	24-30	30-36
	30	Post. br.	2.2	4.2	5.5	5 0
		Ant. br.	2.0	2.7	3.7	4 5
		Back	2.3	3 4	5.7	3.4
		Sad.	2.2	3.8	4.8	5.8

R = Days of measurement in relation to injection time.
A = Actual days of measurement based on feather age.
Figures represent average of right and left feather areas in all cases.

doses, the increased growth rate persisted for a longer period in the saddle than in the other feather areas, indicating a lower threshold for saddle feathers.

In an attempt to obtain normal growth rates for a longer period, larger doses of thyroxin were administered. With successive injections of larger doses, further acceleration of feather growth was obtained (Table II). After 2 administrations of 1.5 mg thyroxin with an interval of 6 days between injections, all areas attained or exceeded feather growth rates observed in normal hens. The posterior breast reached a higher growth rate than the other areas, though the period of acceleration was for a longer period in the saddle.

As a consequence of thyroidectomy in brown Leghorn hens there is a marked reduction in feather growth rates and an alteration in the order of growth-rate relations between the various body areas. A decrease in estrogen production[10] is probably responsible for the

[10] Blivaiss, B. B., and Domm, L. V., *Anat. Rec.*, 1942, **84** (Suppl.), 79.

[11] Wang, H., *Physiol. Zool.*, 1945, **18**, 335.

[12] Blivaiss, B. B., 1946, unpublished data.

assumption in such individuals of a growth-rate order of feather areas typical of gonadectomized males[6,11,12] and females.[12] The feather areas of athyroidic hens following thyroxin administration reveal the characteristic threshold differences.

15510

Relaxation of the Pubic Symphysis in Guinea Pigs Following Injections of Desoxycorticosterone Acetate.*

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The recent reports[1] on the concentration and purification of relaxin have led to a renewal of interest in the relaxation of the pelvis symphysis of guinea pigs. It seems progesterone acts on the uterus to release relaxin, which acts on the pelvis and relaxes the symphysis pubis. Since progesterone and desoxycorticosterone induce similar responses, such as prolongation of life and growth in adrenalectonized animals,[2–4] progestational changes in the uterus[5] and growth of the lobules of the mammary glands,[6] it seems likely that desoxycorticosterone could also induce relaxation of the pelvic symphysis of guinea pigs. This report contains data supporting this view.

Methods. Forty-eight virgin oophorectomized guinea pigs were used in this study. They were injected as outlined below, and after a recovery period of at least 2 weeks, they were sometimes used again for these experiments. The estrogens† in the form of theelin, and stilbestrol, were given in olive and peanut oils, and injected subcutaneously daily, for 2 days. The site of injection was on the shoulder and back, as this area[7] gives less oil cysts than any other area commonly used for injecting oils. Four days after the estrogens were started, progesterone and desoxycorticosterone acetate in olive oil were given, either intramuscularly or intraperitoneally.

Pelvic examination was gently carried out while the guinea pig rested quietly on one's lap or knee. The state of pelvic relaxation was determined by manual manipulation of the tuber ischi; these were held between the middle finger and thumb of each hand, while one of the fingers was pressed against the symphysis to determine the extent of movement present. By this method, there was almost no danger of mechanically forcing the pelvic ligaments to yield. The degree of movement was recorded from one to 4+, and those cases of 2+ or more, were considered to be positive for pelvic relaxation. Since the chief interest was to know whether the pubic symphysis was or was not relaxed, it seemed unnecessary to arrange the response into further gradations.

Fluoroscopic examination was also carried

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1 Hisaw, F. L., Zarrow, M. X., Money, W. L., Talmage, R. V. N., and Abramowitz, A. A., *Endocrinology,* 1944, **34**, 122.

2 Emery, F. E., and Schwabe, E. L., *Endocrinology,* 1936, **20**, 550.

3 Gaunt, R., and Hays, H. W., *Am. J. Physiol.,* 1938, **124**, 767.

4 Schwabe, E. L., and Emery, F. E., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 383.

5 Van Heuverswyn, J., Collins, V. J., Williams, W. L., and Gardner, W. U., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 552.

6 Mixner, J. P., and Turner, C. W., *Endocrinology,* 1942, **30**, 706.

† Appreciation for supplies is recognized as follows: Theelin, Dr. Oliver Kamm, Parke, Davis and Company; progesterone and desoxycorticosterone acetate, Dr. Erwin Schwenk, Schering Corporation.

7 Emery, F. E., Matthews, C. S., and Schwabe, E. L., *J. Lab. and Clin. Med.,* 1942, **27**, 622.

TABLE I.

No. of tests	Estrogens, mg	Progesterone, mg	% positive	No. of tests	Estrogens, mg	Desoxycorticosterone acetate, mg	% positive
10	2 th	1½	70	10	2 th	1½	50
5	5 th	1½	75	5	5 th	1½	75
7	2-S	1½	29	7	2 S	1½	43
10	5 S	1½	40	10	5 S	1½	50
5	5 S	5	100	5	5 S	5	75
			Summary				
37			58	37			57

Under estrogens in Column 2, th—theelin, S—stilbestrol. The per cent of guinea pigs showing a positive relaxation of the pubic symphysis is shown in the last column. Body weight range—300 to 580 g.

out, and this helped to confirm the amount of separation that occurred between the bones at the pubic symphysis.

Results. The dosages of estrogens employed were probably above the threshold necessary to prepare the uterus and pubic symphysis for the action of progesterone and desoxycorticosterone acetate. This is shown in Table I by the fact that the per cent of guinea pigs showing relaxation of the symphysis pubis, induced by 1.5 mg of either progesterone or desoxycorticosterone acetate, was about the same in animals primed with either 2 or 5 mg of estrogens.

It will also be noted in Table I that there is no noticeable difference between progesterone and desoxycorticosterone in bringing about relaxation of the pubic ligaments of guinea pigs.

When the total dose of progesterone, or desoxycorticosterone is 5 mg, the relaxation response is obtained in almost all animals, but the degree of relaxation was not always maximal. Likewise, much smaller doses sometimes produced what may be called a maximal response. The degree of relaxation of the pubic symphysis in some of these guinea pigs was equal to that found in advanced pregnancy, as shown by both manual and fluoroscopic examinations of the pelvis.

Both progesterone and desoxycorticosterone induced changes in the mammary glands, sufficient for lactation, and in some cases, large milk drops were pressed from them. Sometimes this was observed as early as 24 hours following the injection, but it usually occurred later, and as long as 4 days in one case. Although these time intervals also fit the time necessary for relaxation of the pubic symphysis, the 2 phenomena seem unrelated and occur independently of each other.

Discussion. Previously[8] it was found that progesterone could substitute for desoxycorticosterone acetate on an equal weight basis when tested on the growth curve of adrenalectomized rats. But as previously discussed, various investigations have found the dose of progesterone necessary to maintain life in immature adrenalectomized rats, varied from 1 to 4 mg.

In fact in the same volume of *Endocrinology* as the above citation, it was shown[9] that in cats the progestational action of progesterone was about 10 times that of desoxycorticosterone acetate. Changes in blood, sugar, and serum electrolytes also require larger doses than needed to maintain life in adrenalectomized animals.[10,11] In the present study it was sometimes found that in identically treated guinea pigs some were well relaxed while others gave no response. Thus, it is apparent that on a weight basis these 2 compounds vary greatly in their different actions, and therefore, a conservative view of the data, (Table I), seems to be that desoxycorticosterone acetate can substitute for progesterone in the reaction involved in the loosening of the pelvic symphysis of the guinea pig, but that the exact dosages have yet to be determined.

8 Emery, F. E., and Greco, P. A., *Endocrinology*, 1940, **27**, 473.

9 Leathem, J. H., and Crafts, R. C., *Endocrinology*, 1940, **27**, 283.

10 Harrison, H. E., and Harrison, H. C., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 506.

11 Thorn, G. W., Howard, R. P., and Emerson, K., Jr., *J. Clin. Invest.*, 1939, **18**, 449.

The palpatory method for determining the extent of relaxation of the pelvic symphysis is not very precise, although in experienced hands, the movement in the pelvic symphysis is readily felt. Fluoroscopic examination helps in determining a positive response, but it is doubtful if this method is as reliable as palpation. The X-ray has given additional data and, as judged from a recent report,[12] it is quite reliable in detecting changes in the pelvis of mice.

Summary. In oophorectomized guinea pigs primed with estrogens, desoxycorticosterone acetate can substitute for progesterone in bringing about relaxation of the pubic symphysis. Only a few animals showed a degree of relaxation comparable to that obtained in advanced pregnancy. Milk secretion was obtained in many of these guinea pigs.

[12] Hall, K., and Newton, W. H., *J. Physiol.*, 1946, **104**, 346.

15511

Roentgenographic Studies of the Normal Human Gallbladder.

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This preliminary study was made to ascertain how the diminution in size of the gallbladder shadow is accomplished following a fatty meal.

Method. Twelve healthy individuals were chosen, 6 males and 6 females, aged 20 to 27 years. Following a fat-free evening meal, 3 g of Priodax (beta-(4-hydroxy-3,5-diiodophenyl)-alpha-phenyl-propionic acid) was given orally.[1] Only fruit juice, tea, black coffee, and water were allowed until 14 hours later. Then after a cleansing enema the first film (A) was taken. Following this a meal consisting of 8 ounces of cream with yolks of 2 eggs was given. A second film (B) was taken one hour later, and a third (C) 4 hours after the first. All exposures were made in the upright position, in neutral respiratory phase, after the gallbladder had been located by fluoroscopic examination.

Roentgenographic Data. In all instances the gallbladder was clearly visualized on the first films (A). The size of the gallbladder varied in different individuals. The shadow appeared to have a greater density at its most dependent, fundic, portion, decreasing in density toward the uppermost, collic, portion, which was usually indistinctly outlined (Fig. 1-4).

On the second films (B), taken one hour after ingestion of the fatty meal, the gallbladder shadow was invariably present, although in 2 instances it was faintly outlined. In 3 instances the decrease in size of the shadow was negligible. In the remaining 9 instances, the shadow was decreased in size about 10 to 50%. The density of the shadow was usually increased and was more uniform, with clearer outline of the neck (Fig. 1-4).

On the third films (C) taken 4 hours after the fatty meal and 3 hours after the second exposure, the shadow of the gallbladder was present in all instances except one. In 6 instances the shadows decreased in size about 50 to 75% as compared to the first films. In 5 instances there was only slight diminution in size of the shadow, and there was a distinct difference in the density between the fundic and collic portions of the gallbladder, the fundic portion being more dense and more sharply outlined (Fig. 3 and 4). In 3 of these the shadows closely resembled in size and shape those on the first films (Fig. 2 and 4).

Interpretation. The ingestion of the Priodax, according to present knowledge, is followed by the appearance of the radio-

[1] Einsel, I. H., and Einsel, T. H., *Am. J. Digest. Dis.*, 1943, **10**, 206.

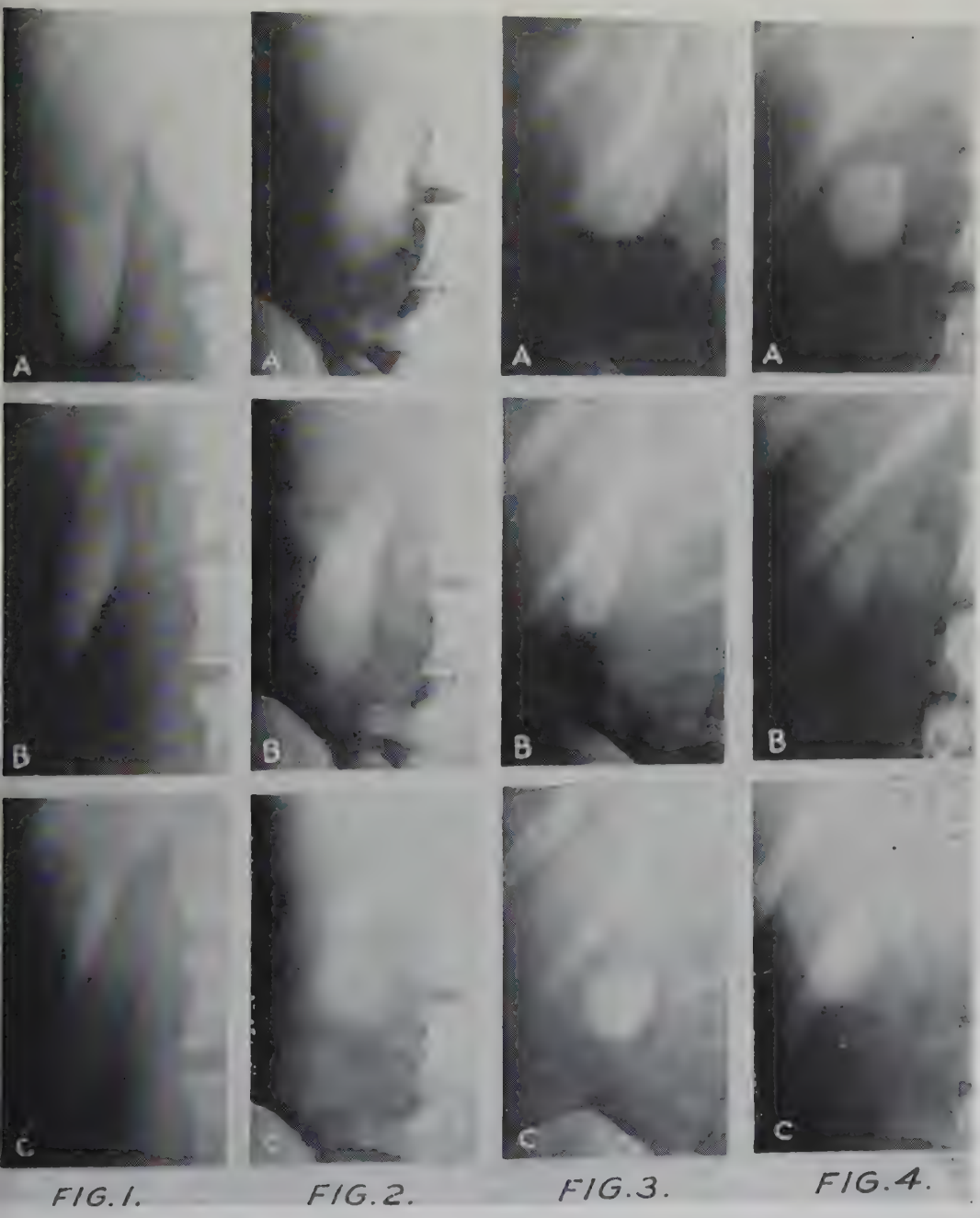

Fig. 1 to 4.

Films A were taken 14 hours after the ingestion of Priodax, B one hour later following the fatty meal, and C 4 hours after the first films. Case 1, 5, 7, and 9, respectively.

opaque substance in the bile. Information is not available as to how long the Priodax is present in the bile coming from the liver and in what concentrations. Experimental data on other substances, methylene blue,[2,3] tetraiodophenolphthalein, eosin, erythrosin and rose bengal,[4] provide information as to the rate of their excretion in the bile and their

accumulation in the gallbladder. According to experiments with one of these substances (methylene blue), the gallbladder can resorb half the volume of its fluid content per hour.[5,6] There is probably a close similarity between the behavior of these substances and that of Priodax. During the period in which radio-opaque substance is present in the bile, some of it reaches the gallbladder. There the opaque substance is resorbed more slowly than the bile and so it eventually attains sufficient concentration to cast a shadow on an X-ray film.

All films in this series were taken in the upright position. The shadow cast by the gallbladder content therefore more closely approximates the anatomic outline of the viscus than that in films taken in the horizontal position.

At the time the first films were taken, neither the hepatic, the common bile, nor the cystic ducts were visualized. Only the gallbladder contained the opaque substance in high enough concentration to cast a shadow. The same situation prevailed at the time of the second and also of the third films. Since in each instance the shadow on the second film changed its contour slightly and its size decreased 10 to 50% of the original and became more dense, it is safe to conclude that practically none of the opaque substance left the gallbladder between the time of the first and second exposures. The presence of sufficient opaque substance to visualize the viscus 4 hours after ingestion of the fatty meal would also support the contention that no loss by evacuation of the gallbladder content through the cystic duct necessarily occurred between the time of the second and third exposures.

Evacuation through the cystic duct of any considerable quantity of the gallbladder content would reduce the amount of radio-opaque substance remaining. Such a decrease of the opaque substance in the gallbladder, however, would cause the shadow not only to become smaller, but also more faint. Selective resorption, on the other hand, removes the solvent leaving the opaque substance in increased concentration, hence the increased density of the shadow on the second films.

A stimulation such as the fatty meal, which causes an increase of the flow of bile from the liver and into the duodenum, may temporarily decrease or increase the flow of bile into the gallbladder. In either event, the gallbladder continues to resorb half the volume of its content per hour. The change in contour, the reduction in size and the increase in density of the shadow as observed on the second films may, therefore, be explained by further concentration of the opaque substance while little or no new bile enters the gallbladder. The changes on the third films, 3 hours after the second, may be explained by the variations in the amount of new bile entering the gallbladder and diluting the opaque substance present. From the data so far obtained it may therefore be concluded that the diminution in size of the gallbladder shadow following the fatty meal is not necessarily caused, as currently believed, by evacuation of the gallbladder content but may be accomplished by selective resorption.

Summary. Cholecystographic studies on 12 healthy individuals were made in the upright position. The data so far obtained indicate that the diminution in size of the gallbladder shadow following the fatty meal may be accomplished not necessarily by evacuation but rather by selective resorption of the gallbladder content.

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2 Halpert, B., and Hanke, M. T., *Am. J. Physiol.*, 1929, **88**, 351.

3 Mills, D. R., and Halpert, B., *Am. J. Physiol.*, 1933, **103**, 265.

4 Halpert, B., and Hanke, M. T., *Am. J. Physiol.*, 1932, **100**, 433.

5 Halpert, B., Thompson, W. R., and Marting, F. L., *Am. J. Physiol.*, 1935, **111**, 31.

6 Halpert, B., O'Connor, P. A., and Thompson, W. R., *Am. J. Physiol.*, 1935, **112**, 383.

Ovulation in Non-Lactating Puerpera.

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Menstruation ordinarily reappears in the nonlactating puerperal woman prior to ovulation. Long after the lochia has ceased, anovular bleeding appears, but usually is followed within a few weeks by ovular menstruation. Griffith and McBride,[1] on the basis of endometrial biopsy studies, reported that ovulation was delayed until 5 months or later postpartum in a group of 11 nonlactating patients. These same women showed anovular menstruation at an average of 2 months postpartum.

The present study was undertaken in order to determine the time of reappearance of ovulation in the nonlactating puerpera by means of the basal body temperature technic.[2] This method of evaluation agrees with direct evidence of ovulation observed at laparotomy.[3] Twenty-three nonlactating puerpera charted their basal temperatures until ovulation and menstruation recurred regularly. Endometrial biopsies were taken during the phase of temperature elevation in 7 of the group, and all of these showed progestational or secretory changes. Menstruation occurred within 2 weeks of the time of biopsy in each case.

As shown in Table I the average time of 8.4 weeks (range 5-15 weeks) for the reappearance of menstruation, agrees with Griffith and McBride's figure of 8 weeks. A significant difference will be noted, however, in the estimated average time of ovulation, which is less accurately determinable. Although the initial menstruation is almost always anovular, a few patients in this series ovulated prior to menstruation, and their data were, obviously, partially responsible for the low figure of 10.2 weeks (range 6-17 weeks) as contrasted with 20 weeks reported by Griffith and McBride for ovulation.

During the course of these studies, it was observed that when lactation was not carried on, or when it was discontinued very early, anovular bleeding was usually seen prior to the return of ovulation. However, after about the eighth postpartum week ovulation occurred with increasing frequency prior to menstruation. These observations will presumably find explanation in the availability of distinct proportions of pituitary gonadotrophins, adequate in some cases to stimulate the normal cyclic growth sequence in the ovary, culminating in ovulation, and deficient in other instances in which the follicular apparatus does not for a time mature and ovulate, but does form enough hormone to bring about cyclic uterine changes. The appearance of ovulation in these instances seems to imitate the succession of events found at the menarche in that the cycles progressively improve until they resemble the normal pattern. Ovulation probably requires more gonadotrophin than menstruation, and the variation in the amount available in different puerpera during the early weeks of the puerperium is relatively large.

Summary. From the study of basal temperatures, correlated with endometrial biopsy

TABLE I.
Reappearance of Ovulation and Menstruation in the Nonlactating Puerpera.

No. of cases	Onset menstruation in avg weeks post-partum	Onset ovulation in avg weeks post-partum
23 (this report)	8.4	10.2
11 (Griffith and McBride)	8.0	20.0

1 Griffith, L. S., and McBride, W. P. L., *J. Mich. M. Soc.*, 1939, **38**, 1064.

2 Rubenstein, B. B., *Endocrinology*, 1938, **22**, 41.

3 Greulich, W., Morris, E. S., and Black, M. E., *Proc. Conf. Prob. Human Fertility*, 1943, p. 37.

control, the average time for the initial ovulation in 23 nonlactating puerpera was found to be 10.2 weeks postpartum. There is considerable variation in the appearance time of ovulation in the absence of lactation. This may be attributed to varying degrees of gonadotrophic stimulation, sufficient in some cases to culminate in ovulation as early as the sixth postpartum week.

15513

Growth Retardation and Corneal Vascularization with Tyrosine and Phenylalanine in a Purified Diet.

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In a previous study dealing with the antagonistic action of certain amino acids toward the growth of *Streptococcus bovis* in a synthetic medium, it was found that a combination of *dl*-phenylalanine and *l*(-)tyrosine showed a marked inhibitory effect.[1] This inhibition of growth could be counteracted by the addition of *l*(-)tryptophane to the medium. Increased amounts of nicotinic acid added to the medium showed no effect. Krehl, Teply, Sarma, and Elvehjem[2] have shown that growth retardation in rats produced by the addition of corn grits to the diet could be counteracted by adding a small amount of tryptophane or nicotinic acid to the diet. In view of this fact the work with amino acid antagonisms was extended in order to determine whether the same phenomenon could be demonstrated in rats.

The following basal ration, similar to that used by Krehl *et al.*[2] was used: Labco vitamin-free casein 10, sucrose 83, corn oil 3, U.S.P. salt I 4, and cystine 0.15 parts. Vitamins were incorporated in the basal ration in the following amounts: thiamine 0.2, riboflavine 0.3, pyridoxine 0.25, calcium pantothenate 2.0, choline chloride 100, inositol 10, 2-methylnaphthaquinone 0.1, biotin 0.01, and folic acid 0.0115 mg per 100 g. Halibut liver oil (diluted 1:2 with corn oil) was fed at a level of 2 drops per week, with α-tocopherol included at 0.5 mg per drop. Additions to this diet were made by replacing an equal weight of sucrose. Substances added to the basal diet were thoroughly mixed with it using a mortar and pestle. Weanling, male rats averaging from 45 to 55 g were used throughout these experiments. Five rats were placed on each diet except in the case of a few control diets in which 3 were used.

The addition of 1% *dl*-phenylalanine and 1% *l*(-)tyrosine to the basal ration produced a substantial growth retardation (Table I) which was accompanied in about 50% of the cases by vascularization of the cornea and marked edematous swelling of the feet. With the addition of levels of phenylalanine and tyrosine as high as 2% each, growth was almost completely inhibited over a 3-week period. At this higher level more severe external lesions were noted and they occurred in 100% of the cases. Spontaneous healing of the external lesions was noted with all of the levels used in these experiments. When 2% phenylalanine and 2% tyrosine were used, the lesions began to appear on the 6th day and healing became apparent on the 15th to 18th day.

Contrary to the results obtained with *Streptococcus bovis*, it was found that the addition of tyrosine or phenylalanine alone to the basal ration produced growth retardation and external lesions to an extent which

[1] Niven, C. F., Jr., and Washburn, Mary R., unpublished.

[2] Krehl, W. A., Teply, L. J., Sarma, P. S., and Elvehjem, C. A., *Science*, 1945, **101**, 489.

TABLE I.
Effect of Nicotinic Acid and Tryptophane on Growth Retardation in Rats on Diets with Added Phenylalanine and Tyrosine.

Ration used	G gained per week* and range
Basal	19 (18-20)
" 1% *dl*-phenylalanine + 1% *l*(−)tyrosine	13 (8-19)
" 1% " + 1% " + 2 mg % nicotinic acid	11 (8-13)
" 1% " + 1% " + 100 " *l*(−)tryptophane	14 (11-18)
" 1% " + 1% " + 500 " nicotinic acid	16 (11-22)
" 1% " + 1% " + 100 " " "	
+ 1% *l*(−)tryptophane	15 (13-17)
" 2% " + 2% "	1 (0.7-2)
" 2% " + 2% " + 100 mg % nicotinic acid	4 (3 -6)
" 2% " + 2% " + 1% *l*(−)tryptophane	4 (2.6-7)
" 2% " + 2% " + 500 mg % nicotinic acid	6 (4.6-7)
" 2% " + 2% " + 2% *l*(−)tryptophane	7 (5 -8)
" 1.5% *l*(−)tyrosine	12 (9-14)
" 4% "	2.4 (2- 3)
" 4% *dl*-phenylalanine	6 (4- 9)
" 100 mg % nicotinic acid	18 (16-19)
" 1% *l*(−)tryptophane	17 (16.6-17.6)

* Average of three weeks.

suggests that the effect produced by the addition of phenylalanine to a diet containing tyrosine may merely add to the effect of the tyrosine. Using deuterium-labeled phenylalanine, Moss and Schoenheimer[3] have shown that when a diet is supplemented with 2% phenylalanine, 20 to 30% of this is converted to tyrosine in the growing rat. The rate of conversion was not affected by the addition of an equal amount of tyrosine to the diet.

Lesions similar to those observed in the present study were previously described by Hueper and Martin[4] as a result of the addition of 10 parts of tyrosine to a diet containing 18 parts of casein and 100 mg % of nicotinic acid.

Corneal vascularization has been seen to occur in a variety of conditions including tryptophane deficiency,[5] lysine and methionine deficiency, and on diets low in protein.[6]

The growth retardation produced by the addition of 1% of phenylalanine and 1% tyrosine could not be counteracted by adding 2 mg % of nicotinic acid or 100 mg % of tryptophane to the diet (Table I). However, a very large amount of nicotinic acid (500 mg %) did appreciably decrease the growth retardation, as well as the appearance of external lesions. There seemed to be no advantage to the simultaneous addition of nicotinic acid and tryptophane.

The almost complete inhibition of growth by the addition of 2% phenylalanine and 2% tyrosine could be alleviated to some extent by the incorporation of 500 mg % of nicotinic acid or 2% of tryptophane to the diet. On the other hand, the addition of nicotinic acid or tryptophane to the basal diet had no growth stimulatory effects.

Two per cent phenylalanine and 2% tyrosine produced no deleterious effects when incorporated in the following diet: Argentine casein 25, Brewer's yeast 6, dried liver 4, sucrose 10, U.S.P. salt I 4, cooked starch 47.9, cellulose 3, and choline 0.1 parts per 100. Mixed tocopherols in corn oil were fed at a level of 11.6 mg per day.

The reason for the rather striking response of the rat toward a relatively low protein diet supplemented with phenylalanine and tyrosine is not known at the present time. It is hoped that experiments now in progress will aid in clarifying some unanswered questions. These experiments, however, serve to demonstrate possible complications arising

[3] Moss, A. R., and Schoenheimer, R., *J. Biol. Chem.*, 1940, **135**, 415.

[4] Hueper, W. C., and Martin, G. J., *Arch. Path.*, 1943, **35**, 685.

[5] Totter, J. R., and Day, P. L., *J. Nutrition*, 1942, **24**, 159.

[6] Sydenstricker, V. P., Hall, W. K., Hock, C. W., and Pund, E. R., *Science*, 1946, **103**, 194.

from diets containing certain amino acids in quantities which are out of proportion to that found in most natural proteins.

Summary. The addition of 1% *dl*-phenylalanine and 1% *l*(-)tyrosine to a purified diet containing 10% casein produced growth retardation and external lesions. Phenylalanine is converted to tyrosine in the animal and so may add to the effect of the tyrosine. The addition of relatively large amounts of nicotinic acid or *l*(-)tryptophane will appreciably alleviate the deleterious effects of these amino acids.

15514

Utilization of Intramuscularly Injected Carotene.

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Carotene administered parenterally has been shown to be poorly utilized.[1,2] Subcutaneous injection of carotene in oil solution[1] or intravenous, intraperitoneal or intrasplenic injections[2] of aqueous colloidal suspensions of carotene have failed to increase stores of vitamin A in the liver of rats. Deficiency symptoms and death have occurred while considerable amounts of the injected carotene were still present in the body.[1,2] Studies in this laboratory of intramuscularly-injected carotene in oil solution have yielded similar results. Carotene in sesame oil, with and without added tocopherols, when injected intramuscularly, has been found to restore growth to depleted rats for a short time only. Upon ensuing death, a considerable fraction of the carotene was found at the site of injection. After being solubilized in water by means of a suitable agent, intramuscularly-injected carotene was as efficiently utilized as carotene administered orally.

Experimental Methods and Results. When carotene is dissolved in Tween 80, (polyoxyalkylene derivative of sorbitan monoleate†) the solution may be diluted indefinitely with water without precipitation of the carotene. A comparative study of the absorption of carotene in Tween 80 solution and in sesame oil solution was made. 0.5 mg of carotene and 0.2 mg of α-tocopherol in 0.1 cc of solvent was injected into the thigh muscles of normal adult rats and the rate at which the carotene passed from the muscle determined. The data are presented in Table I.

The curative effect of intramuscularly-injected carotene as compared with that administered orally was measured by a study of the growth and duration of cure of vitamin A-depleted rats. Seventy male weanling rats were fed the U.S.P. XII vitamin A free diet. After 6 weeks on this diet, when cessation of growth and the appearance of ocular symptoms had marked the depletion of body stores of vitamin A, 40 of the most typically deficient rats were divided by weight into 4 groups. Rats dying within 3 days after receiving the supplement were not included in the data. From a comparison of the growth curves and the data on duration of cure, (Fig. 1) it may be seen that an intramuscular injection of carotene in sesame oil (Group 3) is poorly utilized as compared with the utilization of the same supplement given orally (Group 4). When the carotene was injected in Tween 80 (Group 1) the curative effect was as good as, if not better than, that obtained in the groups receiving carotene orally, either in Tween 80 or oil (Groups 2 and 4).

* The authors wish to acknowledge the technical assistance of the Misses M. Stoneman and F. Shitamae.

[1] Lease, V. G., Lease, E. J., Steenbock, H., and Baumann, C. A., *J. Lab. and Clin. Med.*, 1942, **27**, 502.

[2] Sexton, E. L., Mehl, J. W., and Deuel, H. J., *J. Nutrition*, 1946, **31**, 299.

† Atlas Powder Co.

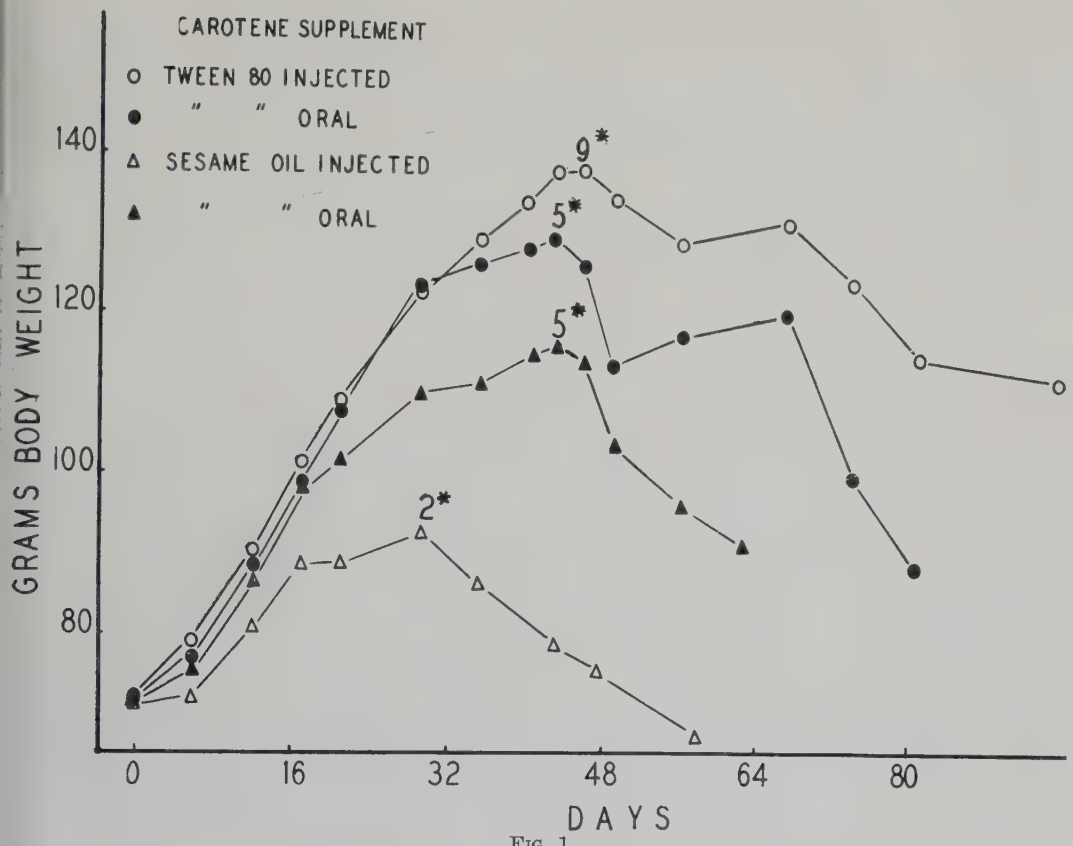

FIG. 1.

Growth of vitamin A depleted rats receiving single doses of carotene in sesame oil and in Tween 80, orally and intramuscularly.

(1) —○— 0.44 mg of carotene + 0.2 mg alpha-tocopherol in 0.1 ml Tween 80 intramuscularly. (9 rats; average survival time—71 days.)
(2) —●— Same as (1) but orally. (8 rats; average survival time—40 days.)
(3) —△— 0.44 mg of carotene + 0.2 mg alpha-tocopherol in 0.1 ml of Sesame oil intramuscularly. (8 rats; average survival time—20 days.)
(4) —▲— Same as (3) but orally. (9 rats; average survival time—40 days.)
* Number of rats alive at point of maximal growth.

The average survival time of the Tween-injected group exceeded that of either of the 2 orally supplemented groups; the time at which maximum growth was attained was about equal for the 3 groups. In Group 3, the carotene injected in oil was the most poorly utilized as judged by all criteria. After the death of each of the rats receiving an intramuscular injection, the thigh muscles were dissected and residual carotene was extracted and determined. The amount of carotene found remaining in the muscle tissue of the oil-injected rats varied from 27 to 76%, while only trace amounts were found in any of the Tween-injected rats.

TABLE I.

Recovery of 0.5 mg of Carotene in 0.1 ml of Tween 80 or Sesame Oil After a Single Injection into the Thigh Muscle of Rats.

Days after injection	% carotene* recovered from muscle	
	Tween 80	Sesame oil
2	7 (43)	98
7	trace (14)	63
15	none (20)	48
29	" (1)	33
37	"	18
65	"	21

* The number in the parentheses indicates the per cent of carotene recovered from subcutaneous tissue near the site of the injection. Only trace amounts were ever found in the subcutaneous tissue in the case of oil injections. Carotene was determined[3] with the Beckman Spectrophotometer.

Discussion and Summary. These experiments show definitely that carotene administered parenterally may be effectively utilized provided the carotene has been water-solubilized by solution in Tween 80. In such a solution not only is there a much more rapid transport of carotene from the site of an intramuscular injection, but there also occurs an effective conversion to vitamin A as demonstrated by the resumption of growth in vitamin A-depleted rats. These findings offer further support to the accumulating evidence[2,4] that the state of dispersion of the carotene is of importance for participation in the physiological processes of absorption, transport and enzymatic conversion to vitamin A.

[3] Tomarelli, R. M., and György, P., *J. Biol. Chem.*, 1945, **161**, 367.

[4] Greaves, J. D., and Schmidt, C. L. A., *Am. J. Physiol.*, 1935, **111**, 492.

15515

Alterations in Body Fluids During Acute Infectious Hepatitis.

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It has long been known that convalescence in acute liver disease may be initiated by a diuresis. Jones and Eaton[1] in noting this phenomenon in an unselected group of patients with acute and subacute liver disease suggested that it may have a favorable prognostic significance. To imply that recovery from liver disease is associated with the production of a large volume of urine, suggests that during the acute phase of the infection there may be profound disturbances in water metabolism involving increased water storage and possibly alterations in the transport and excretion mechanisms. Recognition of the intimate relationship existing between hepatic and renal physiology has impelled clinicians to speak of the ill-defined and little understood "hepato-renal syndrome." The suppression of urine volume in "catarrhal jaundice" and acute yellow atrophy are old observations,[2] as is the oliguria accompanying cirrhosis of the liver, especially when associated with ascites.[3] It is conceivable, therefore, that the latter may in part represent a more serious extension of the same mechanism to an advanced period in the development of chronic liver disease. Indeed, recent evidence suggests that the movement of body fluids during liver disease may be under endocrine control.[4] No available data exist that reveal the precise alterations of the fluid compartments of the body during the course of acute liver disease. Jones and Eaton[1] suggested that the observed diuresis of convalescence was a consequence of improved hepatic efficiency, resulting in a "shift of fluid from the tissues or serous cavities to the blood stream with the ultimate establishment of diuresis." Since adequate methods now exist for the simultaneous determination of approximate interstitial fluid (thiocyanate space), plasma volume and total blood volume, it was believed that a study of the distribution of body fluids during the course of acute infectious hepatitis would be of profit. In this disease one may work in a rapidly shifting metabolic scene.

Materials and Methods. Fourteen patients were studied as part of a group of acute infectious hepatitis cases observed in the Rockefeller Hospital.[5] All were young, adult males

* Dr. Hoagland died Aug. 2, 1946.

[1] Jones, C. M., and Eaton, F. B., *New Eng. J. Med.*, 1935, **213**, 907.

[2] Van Noorden, C., *Pathology*, 1907, **2**, 267.

[3] Adler, A., *Klin. Woch.*, 1923, **2**, 1980.

[4] Ralli, E. P., Robson, J. S., Clarke, D., and Hoagland, C. L., *J. Clin. Invest.*, 1945, **24**, 316.

[5] Hoagland, C. L., and Shank, R. E., *J. A. M. A.*, 1946, **130**, 615.

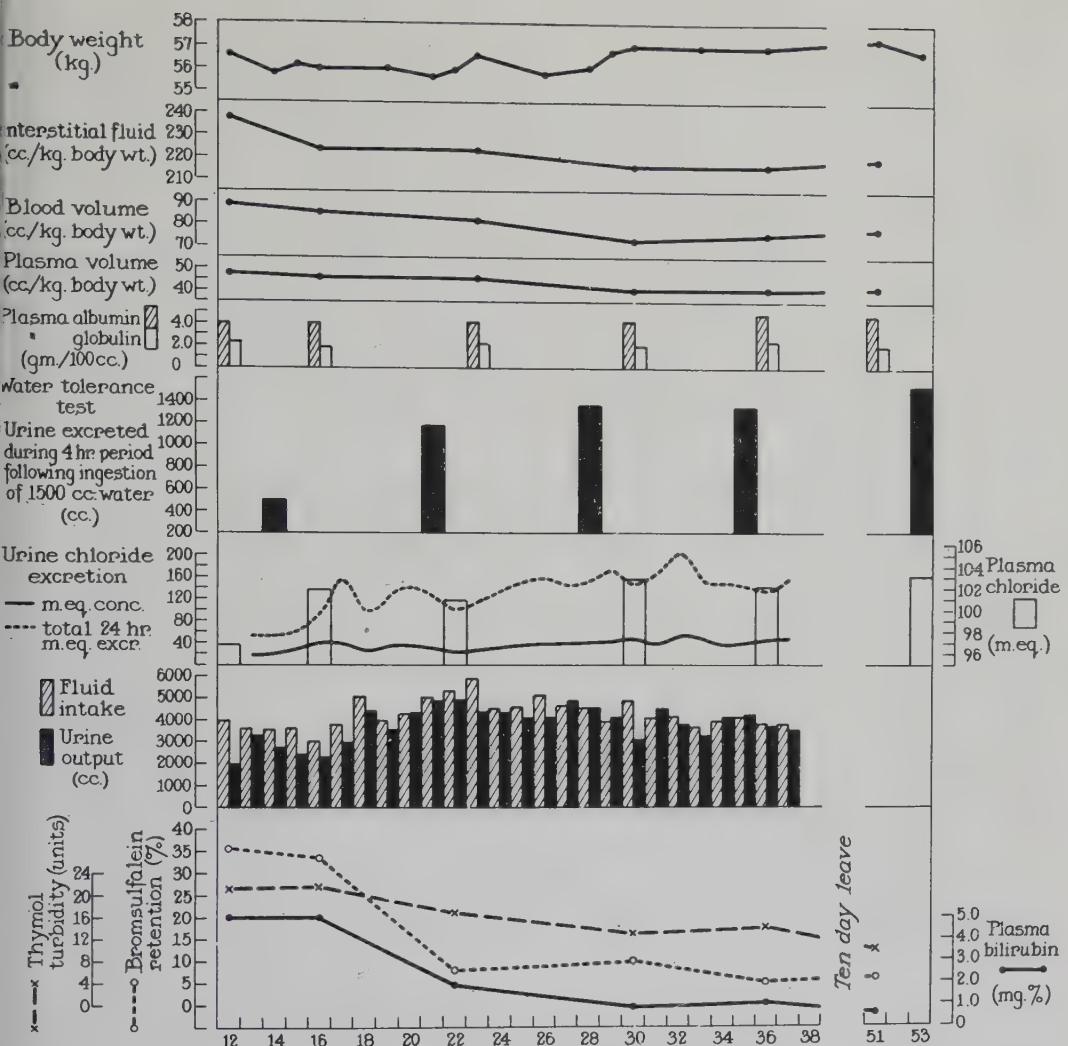

CHART 1.

with previous good health, whose disease course proceeded without complication. In each case, the diagnosis was obtained by a careful history and physical examination and confirmed by appropriate liver function tests. Throughout the study rigid water-balance conditions were maintained. This consisted of the daily determination of body weight and careful measurements of fluid intake and urine output. An attempt was made to keep the diet and salt intake constant for each patient. Measurements of plasma volume and interstitial fluid were determined at 4- to 7-day intervals, according to the simultaneous method of Gregerson and Stewart[6] in which the dye T1824 and sodium thiocyanate measured in the Coleman, Jr. spectrophotometer[7] were used. In this study the interstitial fluid volume was derived by subtracting the plasma volume from the total thiocyanate space. Although this fails to account for the small amount of red blood cell water, there was

6 Gregerson, M. I., and Stewart, J. D., *Am. J. Physiol.*, 1939, **125**, 142.

7 Gibson, J. G., Jr., and Evans, W. A., Jr., *J. Clin. Invest.*, 1937, **16**, 301.

too little change throughout the course of the disease in the red blood cell volume, as measured by the venous hematocrit, to be of significance.[6] Total blood volume was calculated from the plasma volume by means of the venous hematocrit taken on the same sample.[8] The plasma bilirubin was determined according to the method of Malloy and Evelyn adapted to the Coleman, Jr. spectrophotometer.[9] This, together with the bromsulfalein test,[10] measurements of the plasma protein fractions and the thymol turbidity reaction of the serum,[11] were the indices of choice in following the state of liver function at frequent intervals. The total plasma protein and albumin and globulin fractions were determined by the micro-digestion method, in which Nesslerization[12] was used. Normal values for this method are: total protein, 6.0-8.0 g %; albumin, 3.5-5.0 g %; globulin, 1.5-3.0 g %; A/G ratio, 1.5-3.3. Blood specimens for plasma chlorides were collected under oil and measured according to the method of Wilson and Ball.[13] To disclose any tendency to store fluid, a water tolerance test was utilized at weekly intervals.[14] This is a simple expression of the volume of urine excreted under basal conditions in the 4 hours following the rapid ingestion of 1500 cc of water. This test was not performed on the days that the total volume of blood, plasma and interstitial fluid were measured. Preliminary data in the 14 cases are sufficiently constant so that the detailed presentation of one typical case will serve as a text adequate to illustrate the general quality and direction of movement of the body fluid pattern observed in the entire group.

Results. Chart I represents the serial data obtained from a typical case. The patient was a 34-year-old male, seen on the 12th day of his disease. The prehospital period had been marked by malaise, generalized muscular aches and pains, slight anorexia and nausea. There was one mild attack of vomiting 5 days before admission, but the patient had been able to take adequate food and fluids since that episode. Dark urine had appeared 7 days before and visible icterus 3 days prior to hospital admission. Physical examination revealed conspicuous icterus, and an enlarged tender liver. The patient's hospital course was afebrile and with the prompt institution of bed rest and adequate nutrition, convalescence proceeded without unusual event. Measurements of body weight and fluid balance, as well as determinations of blood and plasma volume and interstitial fluid volume were begun the day following admission. It may be noted from the accompanying chart that during the first 2 hospital days there was a fall of 0.8 kg in body weight. This period and the 2 days that followed were marked by the greatest fall in interstitial fluid volume from 237 cc/kg to 223 cc/kg of body weight. The value of 215 cc/kg attained later during convalescence may be taken as a base line. There were slight changes in the same direction but of less magnitude in the blood and plasma volume. Measurements of liver function revealed great impairment as indicated by greatly elevated values for the total bilirubin, the bromsulfalein test and an abnormal thymol turbidity reaction of the serum. It was impressive that despite an adequate fluid intake the urine volume was small compared to the urine output of later convalescence. At this time also the total plasma chloride as well as the urine chloride concentration and total daily urine chloride excretion were low. A water tolerance test done during the first week of admission yielded only 503 cc of urine after the ingestion of 1500 cc of water. By the 17th day of the disease there developed such a marked increase in urine volume that the patient was eliminating between 85 and 90% of his fluid intake. Commensurate with this, greater amounts of

[8] Keith, N. M., Rowntree, L. G., and Geraghty, J. T., *Arch. Int. Med.*, 1915, **16**, 547.

[9] Malloy, H. T., and Evelyn, K. A., *J. Biol. Chem.*, 1937, **119**, 481.

[10] Mateer, J. G., Baltz, J. I., Marion, D. F., and MacMillan, J. M., *J. A. M. A.*, 1943, **121**, 723.

[11] Shank, R. E., and Hoagland, C. L., *J. Biol. Chem.*, 1946, **162**, 133.

[12] Archibald, R. M., personal communication.

[13] Wilson, D. W., and Ball, E. G., *J. Biol. Chem.*, 1928, **79**, 221.

[14] Aldersberg, D., and Fox, C. L., *Ann. Int. Med.*, 1943, **19**, 642.

chloride appeared in the urine, reaching a peak also on the 17th day, despite the fact that the daily calculated salt intake had been constant (9-10 g) since admission. This same period was further characterized by improvement in liver function. A slight but continued fall in the volume of interstitial fluid, total blood and plasma volumes was noted. On the 21st day of the disease a water tolerance test yielded more than double the previous amount of urine, 1170 cc being obtained. The period from the 21st to the 30th day of disease was characterized by an active diuresis and with this there followed stabilization of the interstitial fluid volume, plasma and blood volumes and a measurable improvement in liver function. Following this period the urine output fell slightly, but plasma chloride values and urine chloride excretion remained at sustained high levels. Little change was noted in the results of the water tolerance tests following the period of diuresis. With the restitution of liver function the initial fall in body weight was overcome and there now appeared a weight gain, despite the fact that the daily caloric intake had been maintained at values between 3000 to 3500 calories since admission. At the time of discharge from the hospital the patient had made a complete recovery, as indicated by maintenance of body weight and restoration of liver function. In addition there had been no significant change in the body fluids, which had been stabilized since the 30th day of the disease. Throughout the period of hospital observation, clinical signs of dehydration were never encountered and no peripheral edema or ascites had been noted.

Discussion. If the convalescent values are used as a base line, it is apparent from the data that a large interstitial fluid compartment exists during the acute phase of infectious hepatitis. In addition there is a tendency to retain or store ingested water, as reflected during this period by the poor urine output of the water tolerance test. That this does not represent inadequate renal function is supported by the fact that numerous routine examinations of the urine for albumin, sugar and formed elements in the sediment were always negative, though no formal renal function tests were performed. In the presence of an increased interstitial fluid compartment, as well as an increased plasma and blood volume, it is difficult to attribute the low urine volume, depressed urine chlorides and tendency to store ingested fluid to simple dehydration.[15] As suspected by Jones and Eaton, this increased interstitial compartment persists until improvement in liver function is manifest. With the mobilization of stored interstitial fluid there is a fall in body weight quite parallel to the amount of fluid lost. A great amount of chloride is apparently withdrawn and resides in this compartment in compliance with the laws of diffusion equilibria. This probably accounts for the low initial plasma chloride and depressed urine chloride excretion. This phenomenon has been previously noted in animals with acute liver injury produced experimentally by means of arsphenamine.[16] When mobilization of the interstitial compartment occurs there is an increase in both urine chloride concentration and total urine chloride excretion. With this adjustment the plasma chloride rises and following the diuresis that may appear at this time, there is less tendency to store ingested water as measured by the water tolerance test. The events of this period are probably set in motion by an improvement in hepatic efficiency. With the establishment of convalescence the body fluid compartments move toward stabilization. There follows a rise in plasma protein values somewhat greater than can be accounted for by the slight fall in plasma volume; this probably reflects improved protein synthesis by the liver. The changes in plasma proteins that may occur independent of alterations in plasma volume have been reported experimentally and are to be found especially where nutritional features are of great importance.[17]

The forces underlying the dynamics of

15 Lyons, R. H., Jacobson, S. D., and Avery, N. L., *Am. Heart J.*, 1944, **27**, 353.

16 Soffer, L. J., Dantes, A. D., and Sobotka, H., *Arch. Int. Med.*, 1937, **60**, 509.

17 Mellors, R. C., Muntwyler, E., Mautz, F. R., and Abbott, W. E., *J. Biol. Chem.*, 1942, **144**, 785.

this changing fluid pattern may be suggested in outline. That there may be endocrine factors at work, such as an antidiuretic substance not effectively inactivated by a poorly functioning liver has been indicated.[4] The role of the pituitary in this mechanism has been suggested,[18,19] and requires further elucidation. Recent data, however, disclose a significant elevation in the level of excretion of biologically active estrogen in the urine during acute infectious hepatitis which attains normal values during late convalescence.[20] It is conceivable that movements in body fluid may follow these alterations in active estrogen and be mediated through a central agency such as the pituitary.

Summary. Interstitial fluid volume (thiocyanate space), total blood volume and plasma volume were measured at frequent intervals in 14 young adult males with acute infectious hepatitis. In each patient, the body fluid movement pattern was similar. Detailed data presented in one typical case disclose an increase in the interstitial fluid compartment during the preconvalescent period and a tendency to store ingested water as measured by a water tolerance test. Diminished plasma and urinary chloride values are also found during this period, probably because of chloride storage in the increased interstitial fluid compartment. With convalescence and the restitution of liver function, the interstitial fluid and its retained chloride are mobilized and the interstitial fluid volume falls to lower values. There is an accompanying fall in body weight and frequently a diuresis follows. Less tendency to store ingested fluid is also observed at this time. Plasma chlorides are now restored and urinary chlorides appear in normal amounts. A slight drop in plasma volume and total blood volume accompany these changes and appear to be independent of alterations in the plasma proteins.

It is suggested that these movements in the body fluids may have an endocrine basis.

18 Gilman, A., and Goodman, L., *J. Physiol.*, 1937, **90**, 113.

19 Ingram, W. R., Ladd, L., and Benbow, J. T., *Am. J. Physiol.*, 1939, **127**, 544.

20 Gilder, H., and Hoagland, C. L., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 62.

15516

Recovery Curves of Intraventricular Conduction; QRS Aberration.

GEORGE DECHERD AND ARTHUR RUSKIN.

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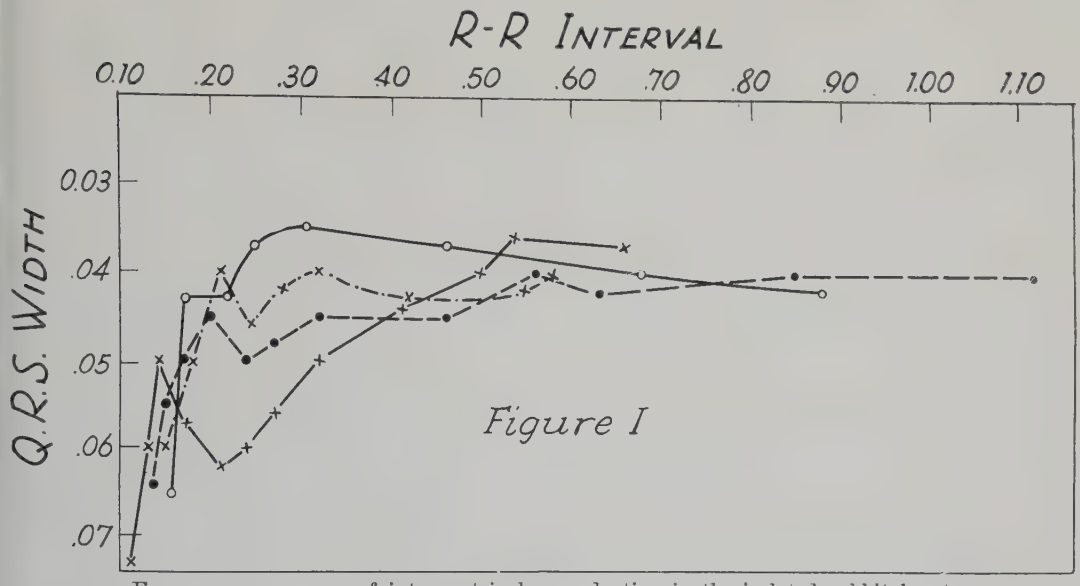
Aberrant intraventricular conduction, with change in form and duration of the QRS complex, is commonly encountered in paroxysmal tachycardia of supraventricular origin, and in premature beats from similarly located foci. Such aberration is regarded as being due to fatigue, or to inadequate recovery, of the conducting tissues. The recovery curves of A-V conduction have been carefully studied;[1] similar studies have been made of ventricular muscle strips, but no analogous data are available for the intact mammalian ventricle.

Method. We have used the isolated rabbit heart, perfused through its coronary vessels by a cannula tied into the aorta. The perfusion fluid was prepared according to the formula of Krebs and Henseleit;[2] it was kept at 37°C, and through it was bubbled oxygen containing 5% CO_2. The atria were cut off, and stimulating electrodes were pushed through the upper part of the I-V septum. The electrodes were activated by an electronic stimulator devised, and made for us, by Dr. S. A. Peoples.[3] This device permits variation in the strength of the stimulus, as well as wide

1 Decherd, G. M., and Ruskin, A., *Brit. Heart J.*, 1946, **8**, 6.

2 Krebs, H. A., and Henseleit, K., *Z. f. Physiol. Chem.*, 1932, **210**, 33.

3 Peoples, S. A., in press.



Four recovery curves of intraventricular conduction in the isolated rabbit heart.

variation in the rate. The overflow of the perfusate ran into a beaker, kept at a constant level by suction, and was returned to the reservoir. Electrodes for the electrocardiographic records were placed in this beaker. Stimuli were delivered to the ventricle at a rate first approximating the idioventricular rate; they were increased by stages up to about 400 per minute, or until some were blocked, or until transient paroxysmal ventricular tachycardia developed.

The QRS width was measured with the Cambridge Record Measuring Instrument. This was plotted against the time interval between stimuli, which was taken as an index of recovery time. Satisfactory preparations beat well for several hours; the experimental data were usually obtained within 60 minutes. Effects of fatigue were further excluded by repetition of both control and drug measurements at various intervals.

Results. Effect of varying rates. Several typical examples of the curves obtained are shown in Fig. 1. Some recovery curves show, in the range of slow rates, gradual shortening of the QRS with increasing rates; more common, however, is gradual prolongation with more rapid rates. A consistent observation is improvement in intraventricular conduction over a short range of rapid rates, causing a notch in the descending limb of the curve where the RR intervals are shortest. Immediately following the notch, however, with increasing rates, QRS widening becomes most marked.

A transient increase in aberration was usually noted in the second, third, and sometimes the fourth complexes of groups produced by sudden increases in rate; rapid shortening of relative refractoriness, as the rapid rate persisted, led to decrease in QRS width and distortion, with the maintenance of a constant QRS form and time for that rate. Similar findings have been made by Scherf[4] in experimental A-V block, and may be seen in patients who have paroxysmal tachycardia with partial A-V block.[5]

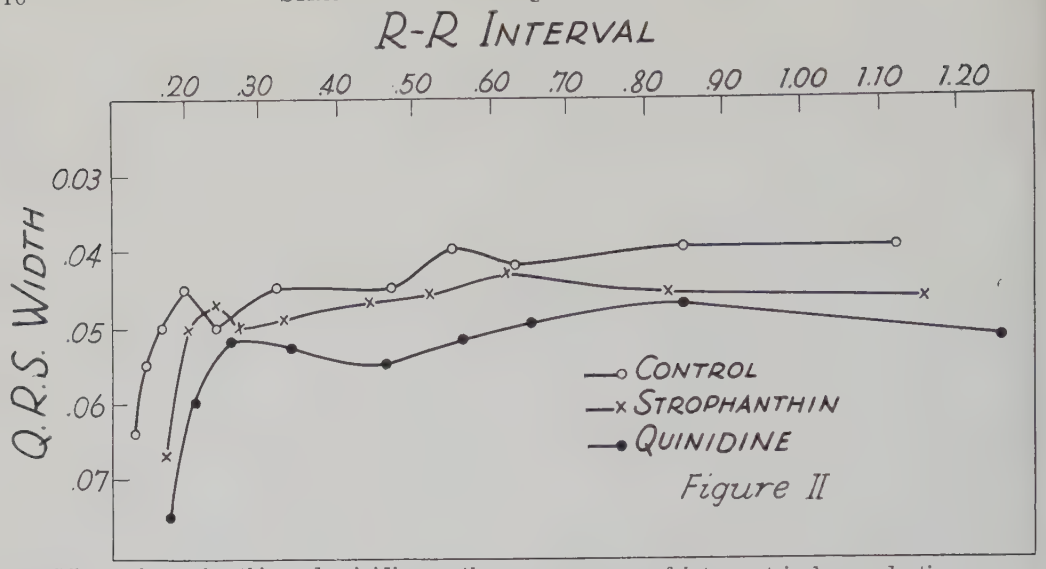
Effect of Strophanthin. Strophanthin-K (Strophosid, Sandoz)* was injected into the rubber connection of the cannula in a dose averaging 0.006 mg/kilo of weight of the intact rabbit. This caused a prolongation of the intraventricular conduction time (Fig. 2), which was greater with larger doses of the drug. This effect disappeared in approximately 15-30 minutes, with a return to the control values.

Effect of Quinidine. Quinidine sulphate was injected in a dose of 0.15 mg/kilo. Pro-

[4] Scherf, D., *Wien. Arch. inn. Med.*, 1929, **18**, 403.

[5] Decherd, G. M., Herrmann, G. R., and Schwab, E. H., *Am. Heart J.*, 1943, **26**, 446.

* These studies were supported, in part, by a grant from the Sandoz Chemical Works, Inc.



Effects of strophanthin and quinidine on the recovery curve of intraventricular conduction.

longation of conduction times, and of refractoriness, was uniformly produced.

Comment. With shortened periods of recovery the intraventricular conduction time is prolonged. This parallels the well-established recovery curve for A-V conduction. There is, however, one exception; at rapid rates there is a narrow range within which I-V conduction is temporarily improved. This phenomenon has not been noted in recovery curves of A-V conduction, unless it is analogous to the period of supernormal conduction sometimes found at the end of the period of relative refractoriness. Another possible explanation lies in the manner in which these values were determined. Recovery curves of A-V conduction are usually drawn from PR intervals of single complexes of varying degrees of prematurity, with a constant basic rhythm. Our own unpublished data, as well as Lewis and Master's,[6] show shortening of refractoriness and improvement of conduction with increasing rates. We have not measured, for the curves shown in the figures, the first few more aberrant complexes following rapid stimulation, but have utilized measurements made in complexes which represented a steady state of relative refractoriness at a given rate. The present data thus correspond to those which might be obtained from paroxysmal supraventricular tachycardia, rather than from single premature beats.

Aberrant wide QRS complexes are often seen after quinidine administration; the effect of digitalis is variable,[7] but aberration may occur. The effect of these drugs on conduction in the ventricle is, therefore, similar to their effect on the A-V conduction tissues. This impairment in conduction is probably effective throughout the myocardium, and cannot be assumed to pertain to clinical or experimental bundle branch block, where there is thought to be a localized region of defective conduction.

Rapid rates of stimulation of the ventricle, usually in the neighborhood of 350-400 per minute, fairly constant in the same, but varying in different hearts, often led to the persistence of ventricular tachycardia after cessation of the stimuli. The duration varied from a few seconds to a few minutes. In this preparation, and under the conditions thus far employed, we have been unable to note any effect of strophanthin in facilitating, or of quinidine in preventing or terminating, these paroxysms.

Summary. Data have been obtained from the isolated rabbit ventricle, from which have been drawn recovery curves of intraventricular conduction. Similar curves have been drawn after the injection of strophanthin and of quinidine.

[6] Lewis, T., and Master, A. M., *Heart*, 1925, **12**, 209.

[7] Berliner, K., *Am. Heart J.*, 1931, **7**, 189.

15517

Electrical Systole (Q-T Interval) of Rabbit Heart.

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The QT interval of the electrocardiogram encompasses the whole of activation and recovery of the ventricular muscle. Its duration varies with the heart rate. Logarithmic curves relating the QT interval to the preceding cycle length have been drawn by numerous authors;[1,2] a recent paper[3] attempts to establish a linear QT-RR relationship. The QT interval is prolonged in many varieties of heart disease, by myocardial anoxemia, by diminution in the blood calcium level, and by quinidine. Administration of digitalis shortens the QT interval, particularly when it has been prolonged by myocardial disease. Robb[4] has recently suggested that extra-cardiac factors may reflexly influence the QT duration.

In human patients, or in intact animals, the feasible variations in heart rate are limited. Hence we have studied the QT-RR relationship in the isolated perfused rabbit ventricle, using the technic previously described,[5] which permits variation in rate from that of the idioventricular pacemaker, 50-100/min., up to 400/min. or more. Strophanthin was injected into the perfusion system in doses approximating 0.006 mg/kilo of intact rabbit; then quinidine was employed in a dose of 0.15 mg/kilo. The effect of these drugs on electrical systole was studied.

Results. The figures show the forms of QT-RR curves obtained from this preparation. The QT interval is often prolonged by changes from very slow to moderate rates; further increasing rates uniformly shorten the QT measurement. The curve often flattens out at extremely rapid ventricular rates.

Quinidine, as has often been shown, always prolongs the QT interval markedly (Fig. 1).

The effect of strophanthin-K (Strophosid, Sandoz) was variable. When used in the above dosage, there was in some preparations prolongation of the QT interval; just as often shortening was obtained; several showed no appreciable change from the control curves. It was then found that when small amounts of strophanthin prolonged the QT interval, larger doses shortened it (Fig. 2).*

Comment. The QT-RR curves obviously are not linear, although in the central portion, excluding the slowest and fastest rates, the deviation from linearity may not be great. We have not attempted to derive formulæ that might fit the curves obtained.

If the QT interval measures, in its first part, intraventricular conduction, but in its greater portion, ventricular refractoriness, it is readily understood why increasing rates should shorten the QT duration, up to a limit at which further shortening becomes impossible. It is difficult, however, to explain on a similar basis the occasional slight prolongation of QT at moderate, as compared to very slow rates.

Clark and Mines[6] noted that strophanthin first prolonged, later shortened, the duration of the electrical response of the isolated frog heart. In human subjects, only the shortening has been observed to follow therapeutic dosage of digitalis glucosides. It appears that prolongation of the QT interval has been demonstrated only in isolated hearts; whether or not it may be found early in the course of gradual digitalization of humans has not yet been

1 Albers, D., and Bedbur, W., *Arch. f. Kreislaufforschung*, 1941, **8**, 150.

2 Ashman, R., Proc. Soc. Exp. Biol. and Med., 1939, **40**, 150.

3 Schlamowitz, I., *Am. Heart J.*, 1946, **31**, 329.

4 Robb, J. S., *Fed. Proc.*, 1946, **5**, 87.

5 Decherd, G. M., and Ruskin, A., Proc. Soc. Exp. Biol. and Med., 1946, **63**, 114.

* Since the submission of these papers, we have been able to demonstrate similar effects of small and large doses of Cedilanid (Sandoz) and Scillaren-B (Sandoz), as well as digitoxin.

6 Clark, A. J., and Mines, G. R., *J. Physiol.*, 1913, **47**, vii.

7 Kisch, B., *Strophanthin*, Brooklyn Medical Press, 1944.

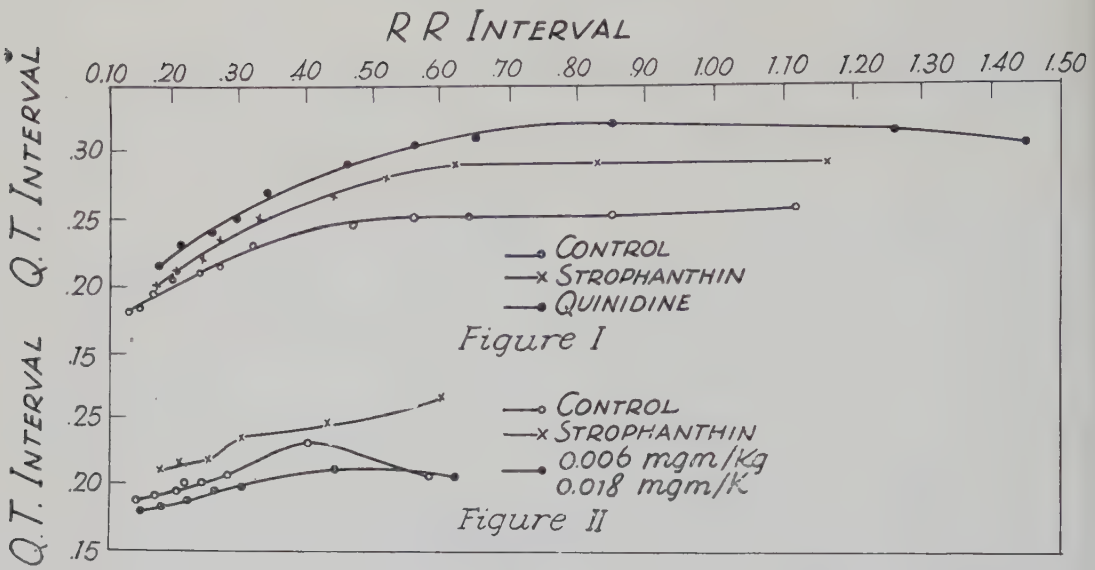

FIG. 1.
Curves relating the QT interval to the cycle length. The effect of digitalis and of quinidine for one experiment is shown.

FIG. 2.
QT-RR curves for a small, and for a large, dose of strophanthin.

determined. Strophanthin has been found,[7] like digitalis, to shorten the QT interval in animal experiments and in human patients. Although the action of quinidine in prolonging refractoriness, *i. e.*, the QT interval, parallels its effect in delaying intraventricular conduction as measured by the width of the QRS complex,[5] this parallelism is lacking for strophanthin, at least in the larger doses.

At fast rates strophanthin and quinidine produce relatively little deviation from the control curve; the effects of increased rates apparently minimize the influence of the drug. Conversely, the most marked changes in the QT interval, produced by these drugs, are usually noted at relatively slower rates of stimulation.

Summary. Curves have been drawn relating the QT interval to the cycle length for the isolated perfused rabbit ventricle. The effect of strophanthin and of quinidine on these curves has been studied.

15518

Effect of Mustard Gas on Mitosis and P^{32} Uptake in Regenerating Liver.*

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Rats (1 month, 40-50 g) were partially hepatectomized and 24 hours later $\beta$is (β-chloroethyl) sulfide ("HS") was injected into the femoral vein. The HS was dissolved in propylene glycol, at a concentration of 2 mg per cc. (A dose of 2 mg per kilo killed rats in 3-4 days.) Ten minutes later P^{32} as Na_2HPO_4 was administered intra-

* The work described in this paper was done at the University of California under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the University of California. It was undertaken at the request of the Chairman of Section 9:5 of the National Defense Research Committee.

TABLE I.

Dose in mg HS	Liver P^{32} as % injected dose	% cells in mitosis
0.1	6.34	
	6.02	
	5.91	
	6.09	0.7
0.2	5.84	
	5.72	
	6.26	
	5.94	0.4
0.4	6.21	
	5.30	
	6.56	
	6.02	0.2
0.20 cc (propylene glycol only)	6.55	
	6.84	
	6.05	
	6.48	1.1

venously in doses not exceeding 1 mg Na_2HPO_4 and 1.5 microcuries per gram body weight. Three hours after the P^{32} injection, the livers were perfused with saline and removed. A small portion of the liver was used for P^{32} assay and the remainder for making a suspension of nuclei to obtain counts of the frequency of nuclei in mitosis. The nuclei were isolated as previously described[1] and the counts made in a counting chamber with Neubauer ruling. A minimum of 2,000 cells was counted for each value given in the tables. The results are given in Table I.

The differences in P^{32} uptake in HS-treated and in control animals are not significant. However, mitosis is markedly depressed and the inhibition of mitosis increases with increasing doses of HS. A dose of 0.1 mg of HS will reduce the mitotic rate to about one-half that of the control, while 0.4 mg reduces the rate to one-fifth of the control. Serial sections were made of the livers which on microscopic examination showed no pathological changes. The depression of the mitotic rate cannot therefore be attributed to a direct necrotizing action of the HS.

In a second series the 0.2 mg HS in propylene glycol was given at 4, 12, 16, and 22 hours after partial hepatectomy. All animals received P^{32} intravenously 24 hours after the partial hepatectomy and the livers were perfused and removed 3 hours later. There were 3-4 animals in each group. The results are summarized in Table II.

When the HS is injected 4 hours after partial hepatectomy, at a time before the cells have been stimulated to active mitosis (Group 1) the mitotic rate remains at the level observed in non-regenerating livers.[2] If the HS is given 22 hours after hepatectomy and allowed to act for 5 hours (Group 5) the mitotic rate is much reduced but not to a level as low as that of Group 1. The data may therefore be taken to indicate that if the HS gets to the cells before the conditions ultimately leading to mitosis are reached, it prevents the establishment of these conditions. The higher mitotic rates of Groups 3 and 4 as compared with Group 1 suggest either local destruction of HS and/or its reaction products or the removal of these substances from the site of action.

The values for percent P^{32} uptake are given with maximum deviations of individual values from the mean of each group. The mean in Group 4 is depressed by one unusually low value which is probably due to an experi-

TABLE II.

Group No.	Hrs after hepatectomy when solution was injected	Hrs with HS	% cells in mitosis	P^{32} %
1	4	23	0.1	3.9 ± 0.2
2	12 (propylene glycol only)	15	0.5	4.2 ± 0.5
3	12	15	0.3	4.2 ± 0.1
4	16	11	0.3	3.1 ± 1.7
5	22	5	0.2	5.0 ± 0.15

[1] Marshak, A., *J. Gen. Physiol.*, 1941, **25**, 275.

[2] Marshak, A., and Byron, R. L., Jr., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 200.

TABLE III.

	10 hrs HS	24 hrs HS	24 hrs cont.	48 hr HS	48 hr cont.
% mitosis	.19	.39	.61	.43	.33

mental error. Group 5 shows a somewhat higher P^{32} content but this is probably not significantly different from Groups 1 and 2.

In a third series, 0.2 mg HS was injected 24 hours after hepatectomy and allowed to act for 10, 24, and 48 hours. Mitotic counts were taken but P^{32} was not followed. Results were as shown in Table III.

Although mitosis is lower in livers examined 24 hours after HS administration than in controls, yet it is higher in those removed 48 hours after HS treatment. The results, as in the previous experiment, suggest that the HS inhibition is temporary, and, upon elimination of HS from the liver, the rate of mitosis increases. Since HS has no effect on the P^{32} uptake of the tissue comparable to that on mitosis, the results suggest that the inhibition observed is not produced by way of the cellular phosphorylating mechanism.

The inhibition of mitosis by HS suggested analogy with similar effects of x-rays which also inhibit mitosis in the nuclear resting stage. A search was therefore made for chromosome aberrations comparable to those found after x-ray treatment. None were observed. In an earlier study, it was shown that treatment with x-rays which also inhibits mitosis in the resting stage produces little if any change in the P^{32} taken up by liver or tumor tissue; however, the distribution of P^{32} between nucleus and cytoplasm was very markedly altered.[4] Obviously, therefore, further work is needed before final conclusions are reached on the effect of HS on the cellular and nuclear metabolism of phosphorus.

Summary. 1. Within 3 hours after intravenous injection, 0.1-0.4 mg of βis (β-chloroethyl) sulfide per 50 g rat produces a marked reduction in the number of cells in metaphase and anaphase in regenerating rat liver. A dose of 0.2 mg inhibits mitosis for at least 24 hours. By 48 hours, this inhibition is released. If the mustard reaches the liver before mitosis is initiated the cells are prevented from entering into mitosis.

2. There is no significant change in P^{32} uptake by liver cells during the interval 3-23 hours after the mustard is injected.

3. Comparison is made with the effects of X-rays on mitosis and P^{32} uptake. No chromosome abnormalities were observed at the dosage levels used.

[4] Marshak, A., *J. Gen. Physiol.*, 1941, **25**, 275.

15519

Application of Gersh's Ferrocyanide Technique to the Study of Experimental Renal Disease.

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Gersh, using histochemical methods, demonstrated in the rabbit, dog, monkey, and other species[1,3] that sodium ferrocyanide was excreted solely by glomerular filtration without apparent tubular reabsorption or secretion;

* Now at the Department of Pediatrics, Baltimore City Hospitals, Baltimore, Maryland.

† The work described in this paper was done with the aid of a grant under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Yale University.

[1] Gersh, I., and Stieglitz, E. J., *Anat. Rec.*, 1933-34, **58**, 349.

[2] Van Slyke, D. D., Hiller, A., Miller, B. F., *Am. J. Physiol.*, 1935, **113**, 611.

[3] *Ibid.*, personal communication from Gersh to the above authors, of p. 613.

these findings have been confirmed by clearance methods.[2] The technic as originally described has been slightly modified and found to be of value in investigating renal function in experimental kidney lesions in dogs and rabbits.

Method. The animal is given intravenously 5 cc per kg of a 10% solution of sodium ferrocyanide [$Na_4Fe(CN)_6 \cdot 10H_2O$]. This solution is injected rapidly. After an interval of approximately 5 minutes, or as soon after the completion of the injection as ferrocyanide is detected in the bladder washings (it may be detected almost immediately after the initiation of the injection in a normal dog, or it may not appear at all, if there is severe renal damage), the animal is lightly anesthetized with intravenous sodium pentothal (15 mg per kg) and the left kidney exposed by a subcostal flank incision, but not manipulated in any way. A second intravenous injection of sodium ferrocyanide solution is given in the same dosage as the first and, simultaneously with its completion, the pedicle of the exposed kidney is clamped and cut and the kidney removed. Sections 1-2 mm thick are immediately cut and, supported on squares of copper screen, are dropped into a beaker of hexane which has been cooled to approximately –80°C by immersion in a bath of solid CO_2 in methyl-cellosolve. After a few minutes the frozen kidney slices are transferred to a dry flask chilled to –80°C. This is then connected to the manifold of a lyophilizing apparatus and the tissues are dehydrated *in vacuo* for 24 hours. The temperature of the flask containing the sections is maintained at the beginning of the dehydration at –25° to –35°C by a bath of equal weights of ice and chilled 95% ethyl alcohol in a Dewar flask and during the last 4 hours of this period the flask containing the kidney slices is allowed to come from approximately –10°C to room temperature. Melted paraffin is added to the warmed flask and the dehydrated tissues are infiltrated with paraffin by evacuation of the air in the flask; sections are cut at 25 micra and mounted without contact with water by gently spreading them on a warmed slide. The mounted sections are then exposed (dropping bottle technic) in succession to saturated solutions of ferric chloride in xylol and absolute alcohol and a 2% solution in 95% ethyl alcohol. Rinsing in 95% ethyl alcohol, dehydration in absolute ethyl alcohol, clearing in xylol and mounting in clarite then follows. A duplicate section may be counterstained by alcoholic eosin after having been run through the alcoholic ferric chloride solutions. In examining the sections that are not counterstained, the use of a substage lamp with a pink filter facilitates the detection of small amounts of the blue ferric ferrocyanide precipitate.

Results. A section prepared in this manner from a normally functioning kidney usually shows a granular blue deposit within Bowman's capsule and a blue-staining of the tuft and capsule itself. At times there may be no deposit, but the blue-staining of the capsule indicates that ferro-cyanide has been filtered. The purpose of the second injection of sodium ferrocyanide is to insure that there will be a deposit of ferric ferrocyanide within Bowman's space; often none can be found here in a kidney with normal function after only one injection, as recommended by Gersh. A blue deposit is detectable within the lumen of the proximal convoluted tubules, or adherent to the margin of their cells. (These tubules often may be somewhat distorted by artefact.) In the remainder of the tubular system the deposit in the lumen is heavier than in the proximal convoluted tubules.

Various experimentally produced renal disturbances have been studied by the use of this method. Acute hydronephrosis was produced by ligation of one of the ureters and the kidneys studied 24 hours later. In the hydronephrotic kidney, ferrocyanide precipitate was plentiful in the glomerular spaces and in most of the proximal convoluted tubules, scanty and only irregularly present in the loops of Henle and distal convoluted tubules and usually absent in the collecting tubules. In the control kidney the precipitate was found throughout the tubular system.

In a dog that had received 3.5 mg per kg of $HgCl_2$ 24 hours previously, the ferrocyanide deposit was plentiful in the glomerular spaces. The necrotic cells of the proximal convoluted tubules were stained diffusely blue but no

precipitate was found in the rest of the tubular system.

The method has also been used in the study of the renal injury produced by intravascular hemolysis and by the injection of solutions of hemoglobin. These results will be reported in detail subsequently.

Summary. Gersh's ferrocyanide histochemical renal function technic, slightly modified, has been found useful in studying experimentally produced renal lesions. In contrast to the finding of Gersh that such preparations fade rapidly, these have shown no change after 9 months.

15520

A Dialyzable Medium for Cultivation of Group A Hemolytic Streptococci.*

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In the course of work with the filtrates of streptococcal cultures a need was felt for a purified medium capable of supporting abundant growth of the organisms. Synthetic media of the present day did not appear to meet the requirements fully since no one of these media is uniformly capable of supporting heavy growth of all pathogenic strains of interest. Ultimately such defined media should be sufficiently developed to supplant the procedures described in this report.

Since it had previously been shown by Stock[1] that some toxin-producing streptococci are capable of growing on a medium containing only dialyzable constituents, a trial was made with heart muscle extract and commercial peptone purified by preliminary dialysis. It was found that the medium made with these purified constituents actually allowed more rapid and abundant growth in most cases than the standard Todd-Hewitt broth[2] of similar composition. The success of these efforts suggested that the medium might find a wider use than had originally been contemplated.

Preparation of the Purified Beef Heart Extract. Beef heart, after being stripped of fat and finely ground, is soaked overnight at 4°C in water,† 250 cc per pound of meat. Following this infusion the mixture is heated to 85°C for 30 minutes. This amount of heating appears to be just sufficient to coagulate muscle proteins and to express intracellular fluid, yet sufficiently mild to spare most of the heat-labile growth factors. A ten-fold reduction in the quantity of meat required is made possible by this minimal exposure to heat; with more violent extraction or with subsequent heat sterilization of the completed medium, a larger quantity of meat is needed.

At the end of the 30-minute heating period, the extract is filtered through fluted filter paper (supported under the point of the cone with cheese-cloth) and the juice expressed with a wooden masher, then cooled by immersion of the receiving flask in running water. The pink, slightly turbid filtrate, now about 300 cc per pound of meat, is introduced into cellophane casings‡ each about one meter in length and suspended in the upper two-thirds of a suitable tall, narrow vessel against 2 vol-

* This investigation was carried out under a contract between the Rockefeller Institute for Medical Research and the Commission on Hemolytic Streptococcal Infections, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, United States Army.

1 Stock, A. H., *J. Immunol.*, 1939, **36**, 489; *J. Biol. Chem.*, 1942, **142**, 777.

2 Todd, E. W., and Hewitt, L. F., *J. Path. and Bact.*, 1932, **35**, 973.

† No difference in the rate or amount of growth was observed when New York City tap water was compared with distilled water.

‡ Visking Cellulose Sausage Casing, "NoJax" 27/32.

TABLE I.
Comparison of Commercial Peptones.

Brand	% dialyzable	pH of dialysate	Order of increasing color of dialysate	Precipitate with phosphate	Order of decreasing value for growth	Cost per lb
Pfanstiehl	79	5.32	9 (B)	±	1	$4.10
Difco proteose	63	6.80	3 (Y)	±	2	6.00
" neopeptone	80	6.90	7 (B)	++	3	6.00
" bactopeptone	89	7.28	4 (Y)	0	4	4.85
" tryptose	78	7.15	5 (Y)	0	5	6.00
" tryptone	85	7.18	6 (Y)	0	6	4.50
Wilson bacteriological	89	5.57	2 (Y)	0	7	1.10
Witte	55	6.70	1 (Y)	++	8	*
Fairchild	85	5.43	8 (B)	0	9	*

B—Brown. Y—Yellow. *Not available.

umes of water outside. After 12-18 hours dialysis at 4°C, the outside water is replaced; this in turn is replaced after a similar interval for a third dialysis. In this way an estimated 90% of the dialyzable components are extracted into a volume six times the volume of the original filtrate.

If the pooled dialysate is not to be used immediately for media, it should next be concentrated *in vacuo* (15-20 mm Hg) to about 1/20 the volume. With care it may be possible to avoid the use of an antifoaming agent in this process; if one is required, it appears preferable to make small additions of ethyl alcohol rather than the more toxic and persistent octyl alcohol customarily employed.

On standing overnight in the ice box, this concentrate yields an abundant crop of creatine and creatinine crystals which may be removed by centrifugation and discarded. No difference could be detected in the quantity of growth supported by the medium when these crystals were eliminated from one portion and retained in another.

The concentrate may be stored for a period of at least eight months, or longer, by freezing in plastic containers in a dry ice box or by freezing and drying in high vacuum. The latter procedure appears to be the one of choice, despite some difficulty in the drying due to the high salt content with resultant hygroscopic nature, since portions of the dried material may readily be weighed to meet the varied requirements of different experiments.

A yield of about 8 g of the dried extract per 454 g (1 lb) of the original meat is obtained. Excellent growth is supported by 1 g of this material per liter of medium, or with frozen preparations, a quantity representing 1/8 lb of meat per liter. These quantities are at least twice the minimum requirements for optimal growth.

Purification of the Peptone. In Table I are listed various properties of different commercial peptones. It will be seen that there are wide differences in their capacities to support growth, in cost and in color, so that in any particular problem the choice must be governed by the relative weight assigned to these factors. It has, moreover, been observed (Fig. 1) that the different peptones cannot be consistently graded on the basis of growth rate and yield since their relative values in this regard are in part dependent on the particular strain of group A streptococcus employed.

An experiment shown in Fig. 1 measured turbidimetrically the growth of 5 strains of group A hemolytic streptococcus in dialysate media made with 9 different commercial peptones. At zero time each tube, containing 9 cc of medium, was inoculated with one loop of a 12-hour broth culture of organisms. Data are shown for the dialysate media (1 to 9) and for the stock Todd-Hewitt medium[2] made with unpurified Pfanstiehl peptone.

Of the peptones studied the 2 most useful for present purposes appear to be Pfanstiehl peptone and Difco Proteose. Both support excellent growth for all strains tested. The lower cost of the former recommends it for large scale uses in which economy is a more important consideration than freedom from colored impurities. These colored ma-

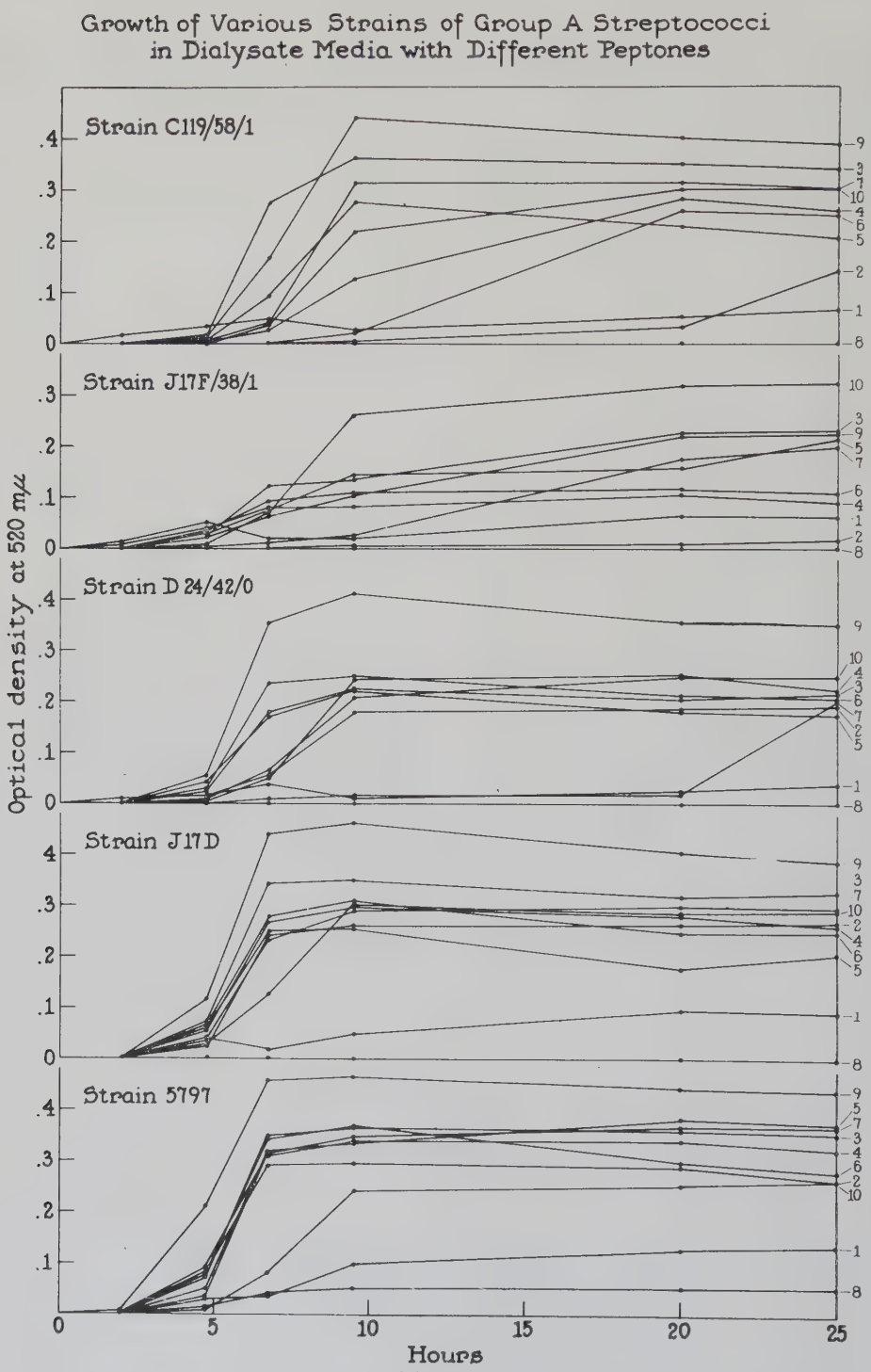

Fig. 1.

Growth of 5 different strains of group A hemolytic streptococci in dialysate media made with the following peptones: 1, Witte; 2, Wilson bacteriological; 3, Difco proteose; 4, Difco bactopeptone; 5, Difco tryptose; 6, Difco tryptone; 7, Difco neopeptone; 8, Fairchild; 9, Pfanstiehl; 10, Stock Todd-Hewitt medium with unpurified Pfanstiehl peptone.

terials can be reduced, but not eliminated, by repeated adsorption with charcoal at different pH's; what remain tend to persist through any subsequent fractionations of the culture fluid and may for this reason be found most objectionable. Proteose peptone, on the other hand, is initially of low color content and can be rendered almost colorless by charcoal adsorption of a 20% solution at pH 8.0. This step also removes the slight precipitate that sometimes develops at this alkalinity.

As a consequence of the high calcium content of neopeptone (Difco), the dialysate medium with this peptone becomes turbid after addition of the phosphate. If removed by preliminary filtration, the turbidity reappears during growth.

For purification the peptone is dissolved in water at 80°C to make a 20% solution, to which activated charcoal about 50 g per liter is added; the mixture is held at 80°C with stirring for one hour, then filtered hot through soft filter paper with the aid of suction. The clear filtrate is then dialyzed in a manner similar to that of beef heart extract.

After completion of dialysis, the pooled dialysate is adjusted to pH 8.0 and again adsorbed with charcoal 50 g per liter, overnight at 4°C. Following removal of the charcoal by filtration, the solution may be concentrated for storage by evaporation *in vacuo,* followed by freezing and drying. It is desirable to prepare as large a quantity of the dried purified peptone as possible in order to assure uniformity of experimental results. There appear to be uncontrolled variations in the qualities of different lots of the same brand of commercial peptone.

Preparation of the Complete Medium (Solid Content 2.3%). The following ingredients per liter of ultimate volume are dissolved in about 900 cc of water, adjusted to pH 8.0 with approximately N/1 NaOH, and then brought to a volume of 950 cc.:

Peptone: 15 g (or the dialysate from 20 g of original peptone)

NaCl: 2 g

Na_2HPO_4 (anhydrous): 0.5 g

This solution may be heat sterilized without impairment of the growth properties, although some undesired color and precipitate may be formed.

Just before use, the medium is completed by addition of 50 cc per liter of final volume of the following mixture, sterilized by filtration:

Beef heart extract: 1 g (or the dialysate from 1/8 pound of meat)

Glucose: 2 g

(Volume adjusted to 50 cc, pH to 8.0 at this point)

$NaHCO_3$: 2 g

Since the whole medium filters at about the same rate as water, it might be preferred to sterilize entirely by filtration. In this case, all ingredients are dissolved in their final concentration (the bicarbonate again being withheld until the pH is adjusted to 8.0) and the lot filtered.

In either case the medium should be inoculated immediately after completion; otherwise the pH may rise to 8.5 or higher in a few hours from decomposition of the bicarbonate. If delay in inoculation is unavoidable, the bicarbonate should be omitted and either added later or the pH during growth controlled by intermittent additions of 18 N NaOH.

It has been found possible, although not for our purposes advantageous, to combine the dried materia'ls in the proper proportions so that complete medium may be formed merely by the addition of water. Such a procedure may be useful when it is necessary to use small amounts of a uniform medium at irregular intervals.

Discussion. Strains C119/58/1 and J17F/38/1, shown in Fig. 1, were selected for study because they had been found by Pappenheimer[3] to be the 2 most difficult to cultivate in the defined medium of Bernheimer, Gillman, Hottle and Pappenheimer.[4] Strains D24/42/0 and J17D were included because they had been found by Wilson[5] to require xanthine. Strain 5797 was currently in use for other purposes and was known to

[3] Pappenheimer, A. M., personal communication.

[4] Bernheimer, A. W., Gillman, W., Hottle, G. A., and Pappenheimer, A. M., *J. Bact.,* 1942, **43**, 495.

[5] Wilson, A. T., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 249.

grow abundantly on stock media. With all 5 of these strains Hartman[6] obtained light growth in the partially defined medium of Adams and Roe[7] which, following Wilson,[5] had been supplemented with horse serum and xanthine.

The dialysate medium evidently contains growth factors not present in sufficient concentrations in either of these semisynthetic media. No attempt has been made to fractionate the natural products further to elucidate the difference.

A potential advantage for the dialysate medium is the reduction in antigenic materials otherwise introduced with unpurified peptone and beef heart. It was found by Todd[8] that horse serum containing strong precipitating antibodies for some antigens in Proteose peptone failed to show any reaction with the corresponding dialysate medium.

Summary. A procedure is described for preparation of a medium containing only 2.3% solid material, all dialyzable. The medium has been found uniformly capable of supporting rapid and heavy growth of group A hemolytic streptococci.

The author is indebted to Dr. Rebecca C. Lancefield and to Miss Dorothy Sloan for their criticism and help.

6 Hartman, T., personal communication.

7 Adams, M. H., and Roe, A. S., *J. Bact.*, 1945, **49**, 401.

8 Todd, E. W., personal communication.

15521

Serum Levels and Excretion Studies in Mice Following Injection of a Penicillin-Albumin Complex.

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In a previous paper[1] mention was made of the fact that penicillin administered intramuscularly to mice in the form of a penicillin-albumin complex was eliminated more slowly than solutions of penicillin in water. Since the publications by Romansky and Rittman[2,3] have shown that penicillin suspended in peanut oil is excreted more slowly than solutions in saline and that when beeswax is added, in concentrations by volume ranging from 0.75 to 6%, the rate of excretion is further reduced, it seemed of interest to compare the efficacy of a penicillin-albumin complex with suspensions of penicillin in oil and in oil-beeswax.

Method. In the studies to be described comparison of serum levels and rates of excretion was made following intramuscular injection of an aqueous solution of a penicillin-albumin complex, suspensions of a calcium salt of penicillin in peanut oil and in peanut oil containing 2, 3 and 5% beeswax* and solutions of penicillin in distilled water.

Since earlier studies on rabbits had shown that serum levels and excretion rates on individual animals frequently varied widely, pooled serum and urine from groups instead of from individual animals were used. The method consisted of the intramuscular injection of penicillin in volumes from 0.2 to 0.5 ml into groups of 30 to 80 mice. The dosage in different experiments ranged from 20 to 400 Oxford units per 20 g mouse. For the excretion studies the first urine col-

1 Chow, B. F., and McKee, C. M., *Science*, 1945, **101**, 67.

2 Romansky, M. J., and Rittman, G. E., *Science*, 1944, **100**, 196.

3 Romansky, M. J., and Rittman, G. E., *Bull. U. S. Army Med. Dept.*, 1944, No. 81, 43.

* These suspensions were kindly supplied by Dr. A. E. Jurist of Products Development Laboratories of E. R. Squibb & Sons. The suspensions were made on a weight volume basis from commercial penicillin.

lection was usually made one-half hour after injection and the second one-half hour later. Thereafter, collections were made at hourly intervals over periods of time ranging from 7 to 14 hours depending upon the nature of the preparation and the dosage given. Each hourly sample consisted of the pooled urine from the total number of mice in the group. The mice were picked up one at a time and the urine collected in a test tube. Urination was usually spontaneous but gentle pressure on the abdomen aided excretion. No special effort was made to empty the bladders of the mice before beginning the experiment. However, handling of the mouse during injection usually caused urination. Early samples were diluted 1-10 with distilled water and passed through Swinney (Seitz type) filters. Later samples were filtered undiluted.

For determination of penicillin concentrations the urine samples were further suitably diluted and tested, first by a 2-fold serial dilution test to determine the approximate concentration, and later by a more precise test in which small amounts (0.1 to 0.03 ml) of suitable dilutions were added to tubes containing 2 ml of a 10^{-6} dilution of a *Staphylococcus aureus* (Heatley) culture. Readings were made on the basis of turbidity after incubation for 18 hours at 37°C, the end point being the highest dilution showing complete inhibition. If, however, there were dilutions showing partial inhibition, the end point was considered to be midway between those showing complete and those showing partial inhibition. The potency was determined by comparison with a standard solution of crystalline penicillin G. By the 2-fold serial dilution test it was possible to determine as little as 0.04 units per ml, but by the more precise test it was not possible to show amounts less than 0.15 to 0.2 units per ml. The highest concentration tested by this method was a 1-20 dilution, while by the serial dilution test it was 1-4.

In the serum level studies the same time intervals for collection were used as in the excretion studies. Five or 6 mice were bled to death from the heart at each time interval and the samples of blood pooled. One-half

TABLE I.
Comparative Rate of Excretion of Penicillin in Urine of Mice Following Intramuscular Injection of a Penicillin-albumin Complex, a Suspension of Penicillin in Peanut Oil, and Solutions of Penicillin in Water.

Hr. after injection	Penicillin-Albumin complex				Cal. salt of penicillin in oil				Crystalline penicillin G aqueous sol. Cal. salt of penicillin aqueous sol.							
	Vol. urine, ml	O.U./ml urine	Total O.U.	%	Vol. urine, ml	O.U./ml urine	Total O.U.	%	Vol. urine, ml	O.U./ml urine	Total O.U.	%	Vol. urine, ml	O.U./ml urine	Total O.U.	%
½	3.3	44	145	19.3	0.85	154	131	17.5	0.35	1076	377	50.3	0.8	519	415	55.3
1	1.2	75	90		0.5	144	72		0.4	218	82.7		0.4	97	38.8	
2	1.6	55	88		1.2	46	55		1.8	22	39.6		2.4	5.6	13.4	
3	.9	39	35		1.2	12.8	15.4		1.3	1.58	2		1.7	0.88	1.5	
4	1.0	21	21		1.3	4.0	5.2		1.4	0.21	0.3		1.7	0.15	0.26	
5	1.0	11.3	11.3		1.0	1.2	1.2		1.7	0.1	0.17		1.4	0.08	0.11	
6	1.2	4.2	5		1.35	0.48	0.65		2.0	0.08	0.16		1.3	0.04	0.05	
7	.9	2.0	1.8		1.4	0.24	0.34		1.2	<0.03			2.0	<0.03	0.06	
			397.1	53			280.79	37.4			506.43	67.5			469.18	62.5

Thirty mice in each group received doses of 25 Oxford units per mouse.

to three-quarters ml could be obtained from each mouse. Blood was collected over periods of 4 to 10 hours depending upon the expected concentration of penicillin. The blood was allowed to clot, was then centrifuged, and the clear serum collected for test. Penicillin assay on the serum samples was done in the same manner as on the urine samples.

The penicillin-albumin complex was made by equilibrating a solution of crystalline penicillin G with human albumin as described by Chow and McKee.[1]

Results. In the first experiments, 4 groups of 30 mice received intramuscular injections of 25 Oxford units per mouse of a penicillin-albumin complex, of a calcium salt of penicillin in peanut oil, of solutions in water of crystalline penicillin G or of a calcium salt of penicillin. Urine was collected over a period of 7 hours and penicillin determinations made. No investigation of serum levels was made. The results are shown in Table I.

From the 2nd to the 7th hour higher concentrations of penicillin were present in the urine of those mice which received the penicillin-albumin complex and the penicillin in oil than in those which received solutions in water. The reverse was true one-half hour after injection. In those mice which had received penicillin in water approximately one-half of the total amount of penicillin injected was excreted during the first half hour.

In experiments with mice it is not possible to collect the total amount of urine excreted but by making frequent collections there is probably little loss. In Table I the volumes collected at the different time intervals and the total excretion in Oxford units is given. When aqueous solutions of penicillin were used and excretion was rapid the total recoveries in the 2 experiments were 67.5 and 62.5%. When a penicillin-albumin complex was used the total recovery was 53%, and when a calcium salt in oil was used recovery was 37.4%. In another experiment, not shown in Table I, in which 400 Oxford units in a 5% beeswax-oil mixture were injected, and excretion was greatly retarded, the total recovery was only 13.7%. It would seem that total recovery of penicillin was reduced as elimination from the body was retarded.

In further experiments a penicillin-albumin complex in 4 different dose levels, 25, 100, 200 and 400 Oxford units per mouse, was compared with like doses of aqueous solutions of penicillin. In all cases the penicillin-albumin complex retarded elimination. In general, like concentrations of penicillin were present in the urine 3 to 4 hours later in those mice injected with the penicillin-albumin complex than in those receiving aqueous solutions of penicillin.

A series of experiments was carried out in which a penicillin-albumin complex was compared with a calcium salt of penicillin in water, in peanut oil, and in peanut oil containing 2, 3 and 5% beeswax. Comparative serum levels and excretion rates were obtained following injection of 400 Oxford units per mouse. All results are expressed in units per ml of serum or urine.

In 2 out of 3 experiments with aqueous solutions, no penicillin was demonstrated in the serum 2 hours after injection; in the third experiment 0.06 unit was still present at this time but none one hour later. With the calcium salt suspension in peanut oil, penicillin (0.33 and 0.17 unit) was still demonstrable in the serum in 2 out of 3 groups at 4 hours; later tests were not made. With the 2% beeswax-oil, the last test was made 5 hours after injection, at which time the serum showed 0.39 unit. With the 3% beeswax-oil, the last test made at 10 hours showed 0.1 unit, and with the 5% beeswax-oil there was 0.17 unit at 9 hours and a trace at 13 hours. With the penicillin-albumin complex, 0.036 unit was present at 5 hours but none at 6 hours. Since it is generally agreed that less than 0.05 unit per ml is an effective inhibiting serum level, neither the oil nor the oil-beeswax experiments were carried out for a sufficient length of time. It would seem, however, that the penicillin-albumin complex was less effective even than 2% beeswax in oil in prolonging effective serum levels.

The figures on excretion indicate that the retarding effect of the penicillin-albumin complex was about the same as that of penicillin in oil. When beeswax was added to

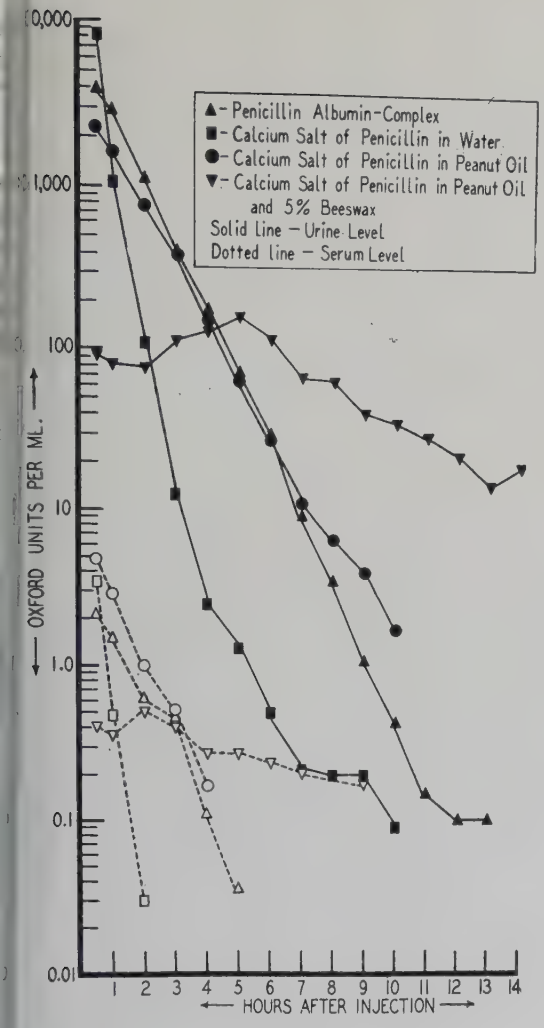

Fig. 1.

the oil the excretion rate was still further retarded, the degree of retardation being increased as the per cent of beeswax was raised. With the penicillin-albumin complex, the suspensions in oil, and solutions in water, the peak of excretion was reached one-half hour after injection. When 2% beeswax was added to the oil the peak in one experiment was still reached at one-half hour but in another experiment the peak was one hour after injection. With 3 and 5% beeswax the peaks of excretion came 3 to 5 hours respectively after injection. As with the serum level studies, experiments were not continued for a sufficient length of time to observe final elimination of penicillin.

Tests made 10 hours after injection of 400 Oxford units of the various preparations gave the following results in units per ml of urine: aqueous penicillin (3 experiments) 0.18, 0.08 and <0.02, suspension in oil (3 experiments) 3.6, 1.5, and 0.15, 2% beeswax-oil (2 experiments) 11.6 and 9.3, 3% beeswax-oil 19.2, 5% beeswax-oil 36.7, and a penicillin-albumin complex 0.44. Tests made 14 hours after injection of the 2, 3 and 5% beeswax-oil suspensions showed 5.7, 10.2 and 19.8 units per ml respectively and 23 hours after injection of 3 and 5% beeswax-oil suspensions 2.8 and 6.6 units respectively were demonstrated. Thus with both 3 and 5% beeswax in oil, penicillin was demonstrable in the urine 23 hours after injection in larger amounts than at 9 to 10 hours after injection of the penicillin-albumin complex. Fig. 1 shows the similarity of blood levels and excretion rates following injection of a penicillin-albumin complex and a suspension of a calcium salt of penicillin in oil. The more rapid elimination when an aqueous solution was injected and the much greater retention when a suspension in oil containing 5% beeswax was used are also shown. Where several experiments were done with the same preparation the figures were averaged.

The results in mice using suspensions of penicillin in oil and in oil-beeswax are in essential agreement with those of Romansky and Rittman who used rabbits as the experimental animals and extended their investigations to man. Richardson, Miller and Ahlgren,[4] using dogs, found slight retardation of absorption when a penicillin suspension in oil was given subcutaneously. Nelson[5] injected 2 human subjects intravenously with 35,000 and 63,000 units of a penicillin-albumin complex and found no prolongation of the action of penicillin. Intramuscular injection in man has, we believe, not been tried.

Summary. A method is described for the use of groups of mice in serum level and excretion studies on penicillin.

A slight prolongation of penicillin in the body of the mouse resulted from the intra-

[4] Richardson, A. P., Miller, I., and Ahlgren, M. W., unpublished.

[5] Nelson, R. A., personal communication.

muscular administration of a penicillin-albumin complex and a suspension of penicillin in peanut oil. When beeswax in 2, 3 and 5% concentration was added to the penicillin-peanut oil mixture the presence of penicillin in the body was greatly prolonged, the degree of prolongation being greater as the concentration of beeswax was increased.

15522 P

Heterophile Antibody Following Administration of Blood Group-Specific Substances.

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(Introduced by David Rapport.)

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In the course of studies on the production of isoagglutinins in human volunteers an agglutinin for sheep cells was encountered in the sera of a number of persons receiving group-specific A substance (Witebsky) and group-specific AB substance (Witebsky).* Twenty-three student volunteers of Tufts College Medical School participated. Sixteen belonging to blood groups B and O received 0.17 to 1.0 mg of A substance intravenously. Blood samples were taken before injection and again 2 weeks after. Sheep cell agglutinins were studied by the method of Paul and Bunnell.[1] An increase in titre of the second serum of 3 tubes or more above the titre of the first was observed in 7 volunteers, 9 remained unchanged or showed less definite increases. Seven volunteers belonging to blood group A received 0.05 mg of AB substance intravenously. Of these only one showed an increase of just 3 tubes.

Since A and AB substances are used to neutralize the isoagglutinins of O blood used for transfusion, the question arose of whether individuals transfused with such blood also developed sheep cell agglutinins. Blood sera were obtained before and one week after transfusion of 15 individuals and compared by the Stuart method.[2] Unlike the student volunteers, the members of this group received A and AB substances combined. Eight belonged to blood groups B and O; of these 5 showed an increase in titre of 3 tubes or more. Seven belonged to blood group A and of these one showed a definite increase.

Volunteers and transfusion recipients whose sheep-cell agglutinin titres were low before injection showed more spectacular rises than those with initially elevated titres. Of 27 individuals, whose original titres were 1/40 or less, 14 showed an increase of 3 tubes or more. Of 11 with original titres above 1/40, only one showed an increase.

The antibody found in these sera differs from the antisheep antibody found in the sera of individuals with infectious mononucleosis in that it can be absorbed with A substance, while the latter cannot. Further attempts to identify this heterophile antibody by absorption with guinea pig kidney and with boiled beef cells have so far yielded equivocal results.

We are indebted to Dr. Ernst Witebsky for valuable advice, and to Miss Lillian Rodofsky for extensive technical assistance.

* The group-specific AB substance was supplied by Eli Lilly & Company; the purified A substance was supplied by Sharp & Dohme.

1 Bray, W. E., *Synopsis of Clinical Laboratory Methods*, pp. 279-281.

2 *Diagnostic Procedures and Reagents*, 2nd Edition, 1945, pp. 449-456.

15523

Effect of Streptomycin on Experimental Infections Produced in Mice with Streptomycin Resistant Strains of *M. tuberculosis var. Hominis.**

Guy P. Youmans and Elizabeth H. Williston. (Introduced by A. A. Day.)
From the Department of Bacteriology, Northwestern University Medical School.

Youmans, Williston, Feldman and Hinshaw[1] have shown that virulent tubercle bacilli may acquire resistance to the *in vitro* bacteriostatic action of streptomycin, either by prolonged exposure to streptomycin in the test tube, or in patients undergoing treatment with this agent. These findings raise the important questions of whether these streptomycin resistant tubercle bacilli are still pathogenic and, if so, whether they are equally resistant to the bacteriostatic action of streptomycin while producing infection in susceptible animal.

Methods. Eighty white mice† weighing approximately 25 g each were divided into 4 groups of 20 mice each. The first group was injected intravenously with 0.1 mg of a suspension of a 21-day-old culture of a streptomycin sensitive H37Rv strain. The second group was injected similarly with 0.1 mg of a 21-day-old culture of an H37Rv strain which had become resistant to more than 1000.0 μg of streptomycin per ml following exposure to streptomycin *in vitro* (designated H37RvR). The third group was injected intravenously with 0.1 mg of a cul-

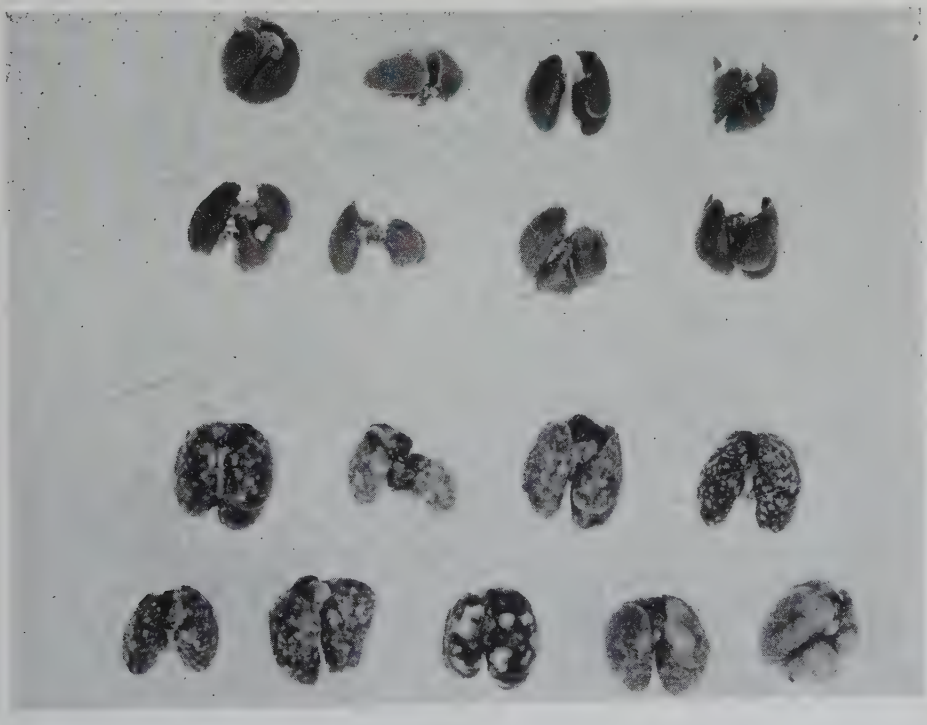

Fig. 1.
Lungs of mice infected with streptomycin sensitive strain H37Rv. Upper 2 rows, streptomycin treated. Lower 2 rows, no treatment.

* This work was aided by a grant from Parke Davis and Company, Detroit, Mich.
The streptomycin was furnished through the courtesy of Dr. L. A. Sweet, Parke, Davis and Company, Detroit, Mich.

1 Youmans, G. P., Williston, E. H., Feldman, W. H., and Hinshaw, H. C., *Proc. Staff Meet. Mayo Clinic*, 1946, **21**, 126.

† Strong A strain.

TABLE I.
Effect of Streptomycin in Mice on Streptomycin Sensitive and Streptomycin Resistant Strains of *M. tuberculosis var. hominis.*

Organism	Streptomycin sensitivity *in vitro* (μg/ml)	Amt streptomycin inj. daily (μg)	No. of mice	No. dead	% mortality	Avg amt of gross pulmonary tuberculosis
1. H37Rv	0.78	3000	8*	0	0.0	None
2. H37Rv	0.78	None	10	6	60.0	+++
3. H37RvR	1000.0	3000	10	10	100.0	++++
4. H37RvR	1000.0	None	10	10	100.0	++++
5. 15	0.78	3000	9†	0	0.0	None
6. 15	0.78	None	9†	6	66.0	+++
7. 67	1000.0	3000	10	5	50.0	+++
8. 67	1000.0	None	9†	5	44.0	+++

* Two mice died day after injection.
† One mouse died day after injection.

ture isolated from a patient with pulmonary tuberculosis (strain 15). The growth of this culture was inhibited *in vitro* by less than 1.0 μg of streptomycin per ml. The fourth group was injected intravenously with 0.1 mg of a culture isolated from the same patient after several months treatment with streptomycin (strain 67). At this time this culture grew readily *in vitro* in a concentration of streptomycin of 1000.0 μg per ml.

Half of the mice in each group served as controls whereas the other 10 mice were

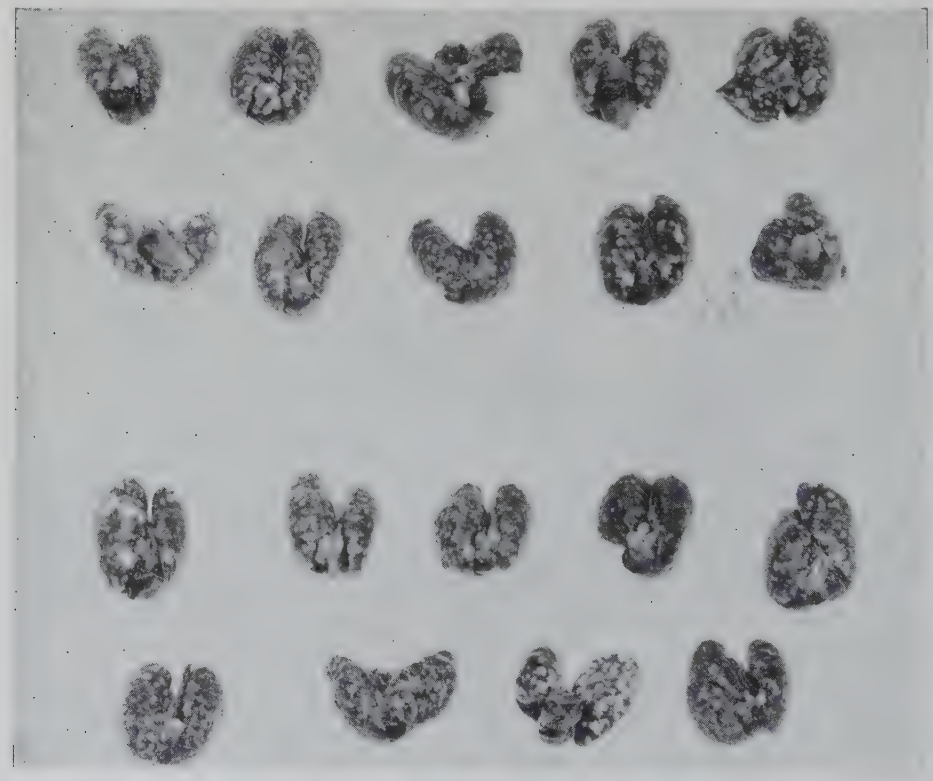

FIG. 2.
Lungs of mice infected with streptomycin resistant H37RvR. Upper 2 rows, streptomycin treated. Lower 2 rows, no treatment.

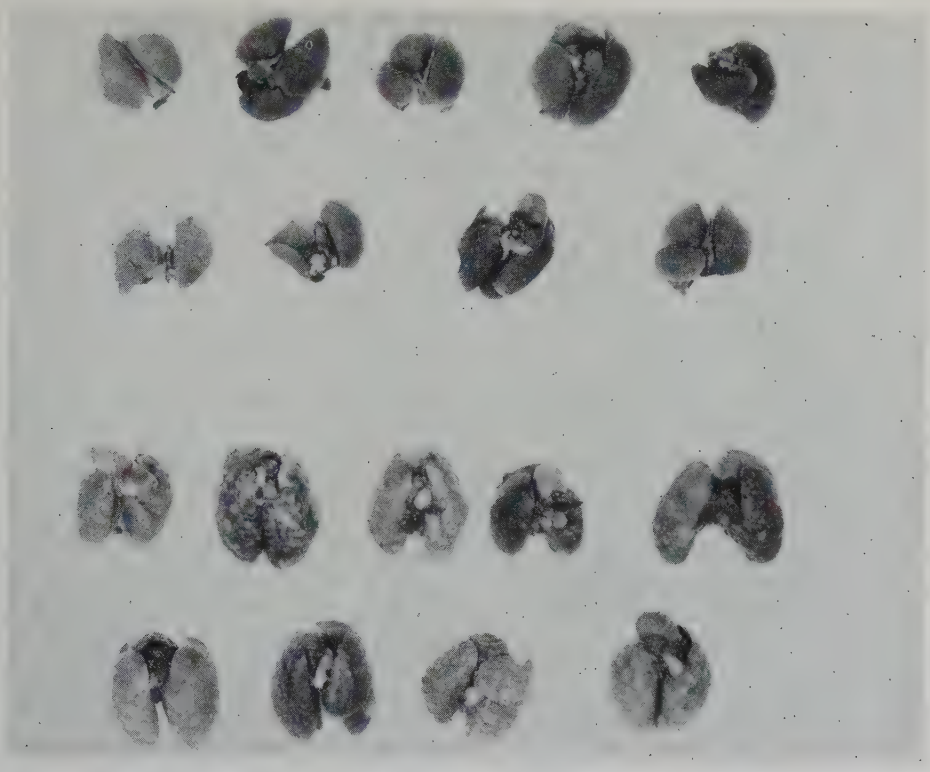

FIG. 3.
Lungs of mice infected with streptomycin sensitive strain 15. Upper 2 rows, streptomycin treated. Lower 2 rows, no treatment.

treated with a total of 3000.0 μg of streptomycin daily, given subcutaneously in 4 equally divided doses at intervals of 6 hours. Treatment was continued for a period of 35 days at which time the surviving mice were sacrificed and observed for the presence of gross tuberculous lesions. Portions of the lungs of 2 mice in each group were cultured for the presence of tubercle bacilli and the cultures obtained were retested for their sensitivity to streptomycin in the manner described previously.[2]

Results. Table I shows the results of the treatment of mice experimentally infected with streptomycin sensitive and resistant cultures of virulent human-type tubercle bacilli. Groups 1 and 5 confirm our previous report[3] that streptomycin exerts a marked suppressive effect on tuberculous infections of mice. All the mice in these 2 groups gained weight over the period of treatment and showed no gross evidence of tuberculosis upon autopsy. Groups 3, 4, 7 and 8 show clearly that not only are the streptomycin resistant cultures virulent for mice but also that streptomycin in the doses used does not influence the course of the infection.

Fig. 1, 2, 3 and 4 show the lungs of the mice. These illustrate graphically the effect of streptomycin in suppressing the infections produced by the streptomycin sensitive tubercle bacilli and the ineffectiveness of streptomycin for the suppression of infections caused by the streptomycin resistant strains.

Tubercle bacilli were reisolated in cultures from the lungs of mice from all 10 groups, though in smaller numbers from those infected with the streptomycin sensitive cultures and treated with streptomycin. The streptomycin

[2] Youmans, G. P., *Quart. Bull. North. Univ. Med. School*, 1945, **19**, 207.

[3] Youmans, G. P., and McCarter, J. C., *Am. Rev. Tuberc.*, 1945, **52**, 432.

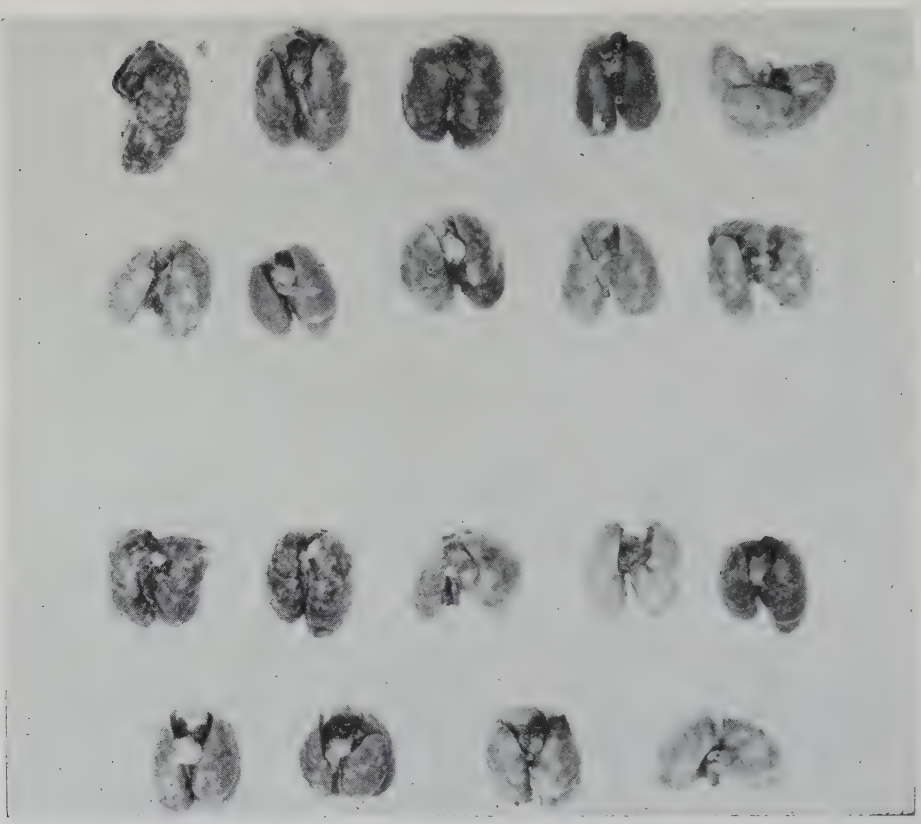

FIG. 4.
Lungs of mice infected with streptomycin resistant strain 67. Upper 2 rows, streptomycin treated. Lower 2 rows, no treatment.

sensitivity of these reisolated cultures was found to be the same as before injection into the mice.

Summary. Streptomycin resistant human type tubercle bacilli were found to be as virulent for white mice as streptomycin sensitive strains. Infection produced in mice with these streptomycin resistant cultures was not suppressed by treatment of the mice with streptomycin.

15524

Variation in Influenza Viruses. A Study of Heat Stability of the Red Cell Agglutinating Factor.*

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For estimating the degree of stability of a variety of materials, both biological and non-biological, it is common practice to utilize elevated temperatures in order to accelerate

* These investigations were aided through the Commission on Influenza, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, United States Army.

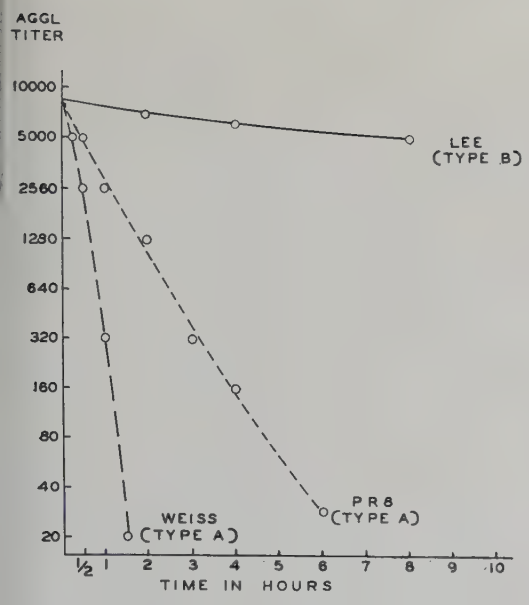

FIG. 1.

Curves showing comparative rates of inactivation at 61.5°C of Lee, PR8, and Weiss strains of influenza virus in untreated allantoic fluids.

the processes that occur slowly at normal temperatures. In view of the slow deterioration at temperatures up to 37°C of the immunizing and hemagglutinating antigens of influenza virus,[1-5] it seemed desirable to define a convenient temperature at which observations could be made rapidly in order to facilitate studies of the relative stability of different preparations of virus or vaccine. In the course of preliminary experiments at 56-65°C it was found that distinct differences exist among strains in terms of the rate of destruction of the red cell agglutinating capacity of the virus. These findings were explored further. It is the purpose of this preliminary communication to present the evidence obtained thus far revealing the existence of wide differences in the stability of hemagglutinin not only among strains but among different lines of the same strain as well.

Materials. Various strains were compared and in some instances the same strain which had been passaged in different hosts, or culture media, was examined. The strains and passage histories will be indicated in the text. The studies reported here have been done on allantoic fluid obtained without red blood cells from infected chick embryos.

Methods. As the source of heat, a water bath was used in which the temperature was accurately controlled within less than 0.1° in the region of 61.5°C This temperature was employed simply because the mercury thermo-regulator was originally set at this temperature when an attempt was made to adjust it to 62°C. Preliminary tests indicated that 62°C was convenient for the majority of strains examined. In some experiments at lower temperatures less well controlled heating equipment was used. For determining stability to heat, the virus suspensions were distributed in 0.5 cc quantities in small rubber-stoppered test tubes and placed in a rack suspended in the bath. At intervals, a tube containing the 0.5 cc aliquot was removed and immersed in iced water. All samples of the same virus suspension were stored in iced water or in the 4°C refrigerator and tested for titer of hemagglutinin at the same time.

The method for estimating hemagglutinating potency has been described.[6] The technic employed involves the use of 0.5 cc quantities of the serially diluted test material and an equal volume of 0.25% suspension of chicken erythrocytes. Serial dilutions were made with an automatic syringe. The endpoint of the titrations is taken to be the highest dilution showing complete agglutination as revealed by the pattern of the sedimented cells. Titers are expressed as the reciprocal of the final dilution of starting material. Procedures providing greater accuracy were not required since gross changes, rather than slight variations, were expected.

Results. The initial observations were made in the course of experiments with the 3 strains of influenza virus which were present in the vaccine recently used by the Army,[2] *i.e.*, the PR8 and Weiss strains of Type A virus and

1 Hirst, G. K., *J. Exp. Med.*, 1942, **76**, 195.

2 Francis, T., Jr., *Am. J. Hyg.*, 1945, **42**, 1.

3 Salk, J. E., Pearson, H. E., Brown, P. N., Smythe, C. J., and Francis, T., Jr., *Am. J. Hyg.*, 1945, **42**, 307.

4 Miller, G. L., *J. Exp. Med.*, 1944, **80**, 507.

5 Stanley, W. M., *J. Exp. Med.*, 1945, **81**, 193.

6 Salk, J. E., *J. Immunol.*, 1944, **49**, 87.

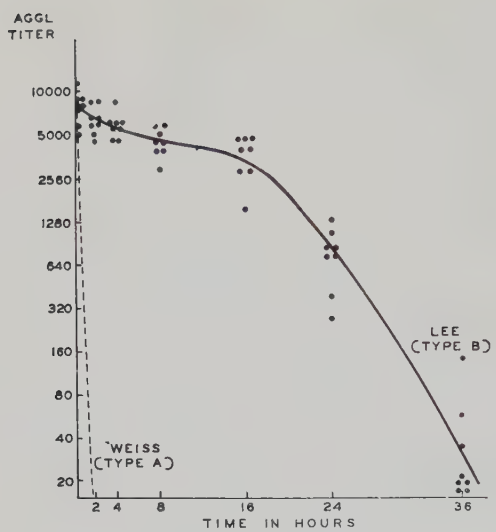

FIG. 2.

Curves showing rates of inactivation at 61.5°C of hemagglutinin of Lee strain of influenza virus Type B in 8 different samples of allantoic fluid and of Weiss strain of Type A virus in 12 different samples of allantoic fluid. (Degree of variation in 8 batches shown on Lee curve. Similar degree of variation along Weiss curve not shown.)

the Lee strain of Type B virus. The PR8 strain had previously been passed through a small number of ferrets, then through 593 mouse transfers, from which it was then introduced into the chick embryo for almost 50 passages. The Weiss strain had come through 3 ferret passages, 32 mouse transfers and 53 egg passages. The Lee strain, after about 8 ferret passages followed by 137 mouse passages, was in the 85th to 90th egg transfer. Fig. 1 shows the results of a typical experiment in which it is seen that the hemagglutinin of the Lee strain was much more stable than those of the PR8 and Weiss strains, and that Weiss was much less stable than PR8. That these variations are related to the different strains of virus and not to individual variations among different preparations of allantoic fluid is suggested by the data shown in Fig. 2. In this experiment 8 different pools of allantoic fluid containing the Lee strain and 12 different pools containing the Weiss strain were compared. While all 12 samples of the Weiss strain had no detectable hemagglutinin after 90 minutes at 61.5°C, little or no change in titer was observed among the 8 samples of the Lee strain in 2 hours. In fact, hemagglutinin of the Lee strain was still detectable after 24 hours, and in some samples after 36 hours.

The difference between the strains illustrated has been observed repeatedly and consistently with successive passages of these particular lines of the respective strains. That the differences are due to properties of the virus and not to some extraneous factor in the allantoic fluid is indicated by examination of the rate of inactivation of the hemagglutinin present in a mixture of the Weiss and Lee strains in allantoic fluid. The admixture with allantoic fluid containing the Weiss strain did not alter the shape of the curve of inactivation of the hemagglutinin of the Lee component. The difference between strains was still evident in preparations in which virus was separated from allantoic fluid by adsorption on red cells and elution[1,7] into a medium other than allantoic fluid, or after adsorption on calcium phosphate[8] and resuspension in physiological solutions. The influence of the composition of the physiological solution on stability will be described elsewhere.

In view of the striking difference in behavior of the Lee strain of Type B virus when compared with the PR8 and Weiss strains of Type A virus, it seemed of interest to determine whether the greater stability of the hemagglutinin was a characteristic of Type B strains. Accordingly, a variety of both A and B strains in allantoic fluid was examined.

A group of 8 Type A strains from different epidemics of the past several years was tested. The prior history of laboratory passage varied, but in all instances the strains exhibited less stability than the PR8 and Weiss strains. Since 61.5°C seemed to be above the critical zone of destruction of the hemagglutinin of these strains, preliminary tests were conducted at 50°C. At this temperature strain difference was evident.

Only 1 of 19 Type B strains tested exhibited a stability curve approximating that of the

[7] Francis, T., Jr., and Salk, J. E., *Science*, 1942, **96**, 499.

[8] Salk, J. E., *Science*, 1945, **101**, 122.

Lee strain. The exception was the "BON" strain isolated in Dr. Burnet's laboratory in Australia in 1943.[9] Of the other 18 strains, all of which had been isolated in chick embryos in this laboratory, two† were obtained from a localized outbreak in May 1945 and the remainder† during the epidemic of 1945-46.[10] The 2 strains from the May 1945 outbreak had been passed 30 times in eggs and all of the 16 strains from the 1945-46 epidemic had been passed less than 10 times. Strain difference was again encountered. The hemagglutinin of all but 3 strains was destroyed in 5 to 15 minutes at 61.5°C. The 3 exceptions (Allen, Goodloe and Hacker), which were more resistant, were among the 16 strains obtained in the 1945-46 epidemic. Although the 2 strains isolated from the May 1945 outbreak had been passed 30 times in eggs, they were no more stable than the majority of the newer strains which had had fewer passages. It would appear from these observations that the particular line of the Lee strain, the stability of which has been described, cannot be considered representative of all Type B strains. Moreover, differences exist in the stability of the hemagglutinating property of strains of virus isolated from the same outbreak.

Since the Lee, PR8 and Weiss strains have had long lines of laboratory passage, it seemed possible that the difference between these and the recent strains might be related to this factor, in part at least. Accordingly, the Lee and PR8 strains of virus which had been maintained in different hosts for varying numbers of passages were transferred to eggs and the allantoic fluids obtained were then tested for stability of their hemagglutinating capacity. Tables I and II show the results of 2 such experiments. From these data it is seen that differences exist in the degree of stability of the hemagglutinin of the different lines of the same strain. In view of the greater stability of preparations having the longest history of egg passage, regardless of previous passage in other media, the following was done to determine the influence of change in host on the stability of the hemagglutinating property of the viruses. The regular egg-passage lines of the PR8 and Lee strains were transferred to mice and carried through 14 passages. After successive passages in mice the virus was returned to the egg and tests were made of the stability of hemagglutinin in the initial egg transfer from mouse material. In the series thus far, passage of the egg-line in mice has not altered the stability of the hemagglutinin of the respective strains of virus. The reverse of this experiment, *i.e.*, passage of the ferret or mouse lines through 27 egg transfers, has not changed the character of the virus in terms of the heat stability of the hemagglutinin.

The persistence of line difference, even after change in host or culture medium, suggests that the host factor is probably of secondary importance and that intrinsic differences of an inheritable nature are involved. The following experiment is cited in further support of the suggestion that the viruses maintained in the separate lines are distinct in terms of heat stability of the hemagglutinin. Chick embryos were inoculated with mixtures of the standard egg-line (Table II, Col. 1) and the ferret egg-line (Table II, Col. 3), combined in varied proportions. Examination of the infected allantoic fluids for heat stability of hemagglutinin indicates that viruses of both lines grew simultaneously and were present in different concentrations depending upon the relative quantities of the respective lines of virus in the different inocula.

Reference has been made to results of other studies that have a bearing on the observations reported here. In Hirst's studies,[1] in which he found that infectivity was destroyed more rapidly than hemagglutinating activity, the PR8 and Lee strains did not differ in the rate at which hemagglutinin was destroyed at 55 and 60°C. Miller[4] followed the rate of decline of infectivity and hemagglutinating

[9] Beveridge, W. I. B., Burnet, F. M., and Williams, S. E., *Australian J. Exp. Biol. and Med. Sci.*, 1944, **22**, 1.

† May, 1945, strains—Chaddick and Ector.

1945-46 strains—Allen, Amdall, Baker, DeSimple, Goodloe, Hacker, Meulder, Mindell, Neubert, Olsen, Orlebeck, Peacock, Potter, Sadowski, Skipton, Solomon.

[10] Francis, T., Jr., Salk, J. E., and Brace, Wm. M., *J. Am. Med. Assn.*, 1946, **131**, 275.

TABLE I.

Effect of Exposure to Temperature of 61.5°C for Varying Intervals on the Hemagglutinin Titers of Allantoic Fluids Containing the PR8 Strain of Influenza Virus Type A from 3 Different Lines of Laboratory Passage.

Time at 61.5° (hr)	Previous laboratory passage		
	Ferret No. 198 593 mice 56 eggs	Ferret No. 198 70 mice 719 tiss. cult. 99 eggs	Ferret No. 198 713 mice 3 eggs
0	20,000+	10,000+	10,000+
¼	10,000+	640	0
½	10,000	160+	0
1	2,560	20+	0
2	640+	0	0
4	80+	0	0

TABLE II.

Effect of Exposure to Temperature of 61.5°C for Varying Intervals on the Hemagglutinin Titers of Allantoic Fluids Containing the Lee Strain of Influenza Virus Type B from 3 Different Lines of Laboratory Passage.

Time at 61.5° (hr)	Previous laboratory passage		
	8 ferrets 137 mice 101 eggs	8 ferrets 338 mice 8 eggs	13 ferrets 0 mice 7 eggs
0	5,000+	10,000+	2,560
¼	5,000+	5,000+	1,280
½	5,000+	5,000+	80+
1	5,000+	2,560	0
2	5,000+	320+	0
4	5,000+	0	0

activity of the PR8 and Lee strains and found in the course of 4 months at 4°C that the Lee strain was less stable than PR8. The observations described in the present report suggest the likelihood that the different findings in different laboratories may be related to the appearance of true variants, with respect to the property in question, that may have developed in the course of passage of the same original strain.

Jones[11] reported the adaptation to heat of the infectious component of influenza virus induced by subjecting the virus-containing suspension to temperatures of 50 to 56°C between successive passages. In personal communication it has been learned that heat resistance was maintained for 3 passages without heat treatment between passages; longer passage without heating has not been studied. It would appear from Jones' studies that the procedure employed resulted in the same effect reported by Armstrong[12] who was able to select a heat-resistant strain of vaccinia virus by passaging samples of virus that had withstood the longest storage periods at 37°C. In Jones' experiments heat-resistant strains did not develop as a result of passage in embryos incubated at temperatures above the range used normally.

Discussion. Evidence for the occurrence of variation in viruses has recently been reviewed by Stanley[13] and by Burnet.[14] Among the plant viruses variants have been shown to possess differences demonstrable chemically and physically as well as biologically.[13] Among the animal viruses the variations that have been described have been in terms of the biological properties of the viruses. For the

[11] Jones, M., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 315.

[12] Armstrong, C., *Pub. Health Rep.*, 1929, **44**, 1183.

[13] Stanley, W. M., *Messenger Lectures in Virus Diseases*, Cornell University Press, 1943, 35.

[14] Burnet, F. M., *Virus as Organism*, Harvard University Press, 1945.

influenza viruses it has been shown that different strains of the same type vary in their antigenic makeup[15] as well as in their infective capacities[16] and adaptability for different hosts;[17] strains of virus differ with respect to toxicity[18] and it appears that the capacity for the O to D transformation[19] described by Burnet also varies. Moreover, the characteristics of chicken red cell adsorption-elution, in terms of temperature and time, are different for the PR8 and Lee strains.[1] Studies of certain biophysical properties of a few strains of influenza virus have been made. The biophysical properties which have been considered are related to particle size and electrical charge.[20] With the exception of suggestive variations in size,[21] differences in the properties of strains have not been reported. It would appear reasonable to consider, as another physical property, the resistance to disruption of the biological activities of the virus. From this viewpoint, the present studies have revealed that strains of influenza virus differ widely in at least one biophysical characteristic; namely, the critical temperature of destruction of hemagglutinating activity. It has been found, moreover, that in these terms different lines of the same strain also vary.

In summary it may be said that the absence of uniform reaction in terms of stability to heat, among different strains of virus, and in fact among different lines of the same strain, suggests another approach to the study of the physico-chemical properties of the influenza viruses as well as studies of inheritable variations in these elementary living units.

The practical implications of the present experiments are pertinent to the problem of vaccine antigenicity and stability. The extension of the observations on the stability of the hemagglutinating capacity of the virus to the question of stability of antigenicity is dependent upon the relationship between the hemagglutinating and immunizing properties. Although conclusive evidence of identity, or lack of identity, has not yet been obtained,[2,5] the trend indicates sufficient parallelism to warrant the suggestion that the stability of the hemagglutinin at elevated temperatures may permit predication of stability of immunizing capacity at lower temperatures. Assuming that the same processes occur at the different temperatures, but at different rates, the technic employed in these studies may be of practical value for rapid determination of the manner in which the virus should be manipulated in order to achieve the most stable vaccine. The fact that strains and lines of virus differ in stability suggests the possibility that with strains or lines of similar antigenic valence, the more stable one may be more desirable in a vaccine. While this would appear self-evident, considering the vaccine in the interval between preparation and inoculation, it remains to be seen whether greater resistance to disruption is advantageous after the vaccine has been inoculated. One could speculate either way; *e.g.*, that the more stable virus would be less liable to destruction before it has reached the antibody-producing centers, or that a more stable virus may be less reactive in antibody formation. To what extent these considerations enter the problem of immunization will have to be investigated.

Summary. A report has been made of the observation of variation among different strains and different lines of the same strain of influenza virus with respect to the heat-stability of the hemagglutinating property. Certain theoretical and practical implications of this observation have been discussed.

The technical assistance of Miss Lee Whyte is gratefully acknowledged.

15 Magill, T. P., and Francis, T., Jr., *Brit. J. Exp. Path.*, 1938, **19**, 273.

16 Francis, T., Jr., and Magill, T. P., *Brit. J. Exp. Path.*, 1938, **19**, 284.

17 Francis, T., Jr., *Trans. and Studies of Coll. of Phys. of Phila.*, 1941, **8**, 218.

18 Henle, W., and Henle, G., *Science*, 1945, **102**, 398.

19 Burnet, F. M., Beveridge, W. I. B., and Bull, D. R., *Austral. J. Exp. Biol. and Med. Sci.*, 1944, **22**, 9.

20 Lauffer, M. A., and Stanley, W. M., *J. Exp. Med.*, 1944, **80**, 507.

21 Beard, J. W., Sharp, D. G., Taylor, A. R., McLean, I. W., Jr., Beard, D., Feller, A. E., and Dingle, J. H., *Southern Med. J.*, 1944, **37**, 313.

15525

Effect of Formalin in Increasing Heat Stability of Influenza Virus Hemagglutinin.*

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A study of the heat stability of influenza virus hemagglutinin was recently reported[1] in which strain and line variations were described. Additional findings of interest have been revealed in further studies employing the same technics. It is the purpose of this communication to report the observation of an increase in heat-stability of the hemagglutinin of influenza virus in the presence of low concentration of formalin, and to discuss certain extensions of the previous observation of strain differences as evidenced by the variable effect of formalin on several strains of virus.

Materials and Methods. The source of virus in these experiments, as before, was the allantoic fluid of chick embyros infected with the various strains. A constant temperature water bath at 61.5°C was used and the method for titrating hemagglutinin is the same as that employed before. The term formalin refers to a solution containing 40% of formaldehyde by volume.

Results. Formalin, in proper concentration, destroys the infectivity of influenza virus, but the immunizing property is retained.[2] Similarly, formalin will destroy infectivity without altering hemagglutinating capacity.[3] Since an excess of formalin will destroy not only infectivity, but other properties of the virus as well,[3,4] it was anticipated that in the presence of progressively increasing concentrations of the chemical there would occur a corresponding increase in rate of destruction of hemagglutinin at any given temperature. It was of interest to find that with 2 of the 3 strains originally studied, the stability of hemagglutinin at 61.5°C was considerably greater in the presence of 0.05% formalin than in the unformalinized control. Table I illustrates the effect observed when the concentration of formalin was varied in allantoic fluids containing the PR8 and Weiss strains of Type A virus and the Lee strain of Type B virus. From these data it is seen that the stability of the hemagglutinin of the PR8 and Weiss strains was enhanced in the presence of the lowest concentrations of formalin used. In contrast, the Lee strain of Type B virus showed a progressive reduction in stability of its hemagglutinin with increasing concentrations of formalin; even the smallest quantity had a deleterious effect. It is of interest that the 2 Type A strains differed with respect to the degree of difference between the untreated and the formalin-treated preparations and that the range of formalin concentration in which the effect was observed was greater for the Weiss than for the PR8 strain.

In view of these differences a number of other strains was examined to see if a type difference was involved. A group of 13 Type B strains was tested. Among these were the BON strain isolated in Doctor Burnet's laboratory in 1943,[5] 2 strains isolated from an outbreak of May 1945 and 10 strains isolated in this laboratory from the epidemic of 1945-46.[6] Some were tested with different quantities of formalin and others were tested only with the 0.05% concentration. In all 13 an increased stability of hemagglutinin was evident in the presence of the formalin. Table II illustrates the effect of different concentra-

* These investigations were aided through the Commission on Influenza, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, United States Army.

1 Salk, J. E., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 134.

2 Smith, W., Andrewes, C. H., and Laidlaw, P. P., *Brit. J. Exp. Path.*, 1935, **16**, 291.

3 Hirst, G. K., *J. Exp. Med.*, 1942, **76**, 195.

4 Eaton, M. D., *J. Immunol.*, 1940, **39**, 43.

5 Beveridge, W. I. B., Burnet, F. M., and Williams, S. E., *Austral. J. Exp. Biol. and Med. Sci.*, 1944, **22**, 1.

6 Francis, T., Jr., Salk, J. E., and Brace, W. M., *J. Am. Med. Assn.*, 1946, **131**, 275.

TABLE I.
Rate of Destruction at 61.5°C of Hemagglutinin of 3 Strains of Influenza Virus in Allantoic Fluid Containing Different Concentrations of Formalin.

Strain and passage	Time at 61.5°C (hr)	Concentrations of formalin, %				
		0	0.05	0.10	0.20	0.50
PR8	0	20,000+	20,000+	20,000+	20,000+	20,000+
(Type A)	.5	10,000	20,000+	20,000+	10,000	0
F-198	1	5,000	10,000+	10,000+	2,560	0
M-593	2.5	640+	5,000+	2,560	0	0
E-56	6	0	640+	0	0	0
	8	0	80+	0	0	0
WEISS	0	10,000+	10,000+	10,000+	10,000+	10,000+
(Type A)	.25	5,000+	10,000	5,000+	5,000+	2,560
F-3	.5	1,280+	5,000+	5,000+	5,000+	40
M-32	1	160+	5,000+	5,000+	5,000+	0
E-54	2	0	5,000+	5,000+	2,560+	0
	4	0	1,280	2,560+	640	0
	8	0	640	640	80	0
LEE	0	10,000+	10,000+	10,000+	10,000+	10,000+
(Type B)	1.5	10,000	10,000	160+	0	0
F-8	3	10,000	1,280	0	0	0
M-137	6	5,000+	0	0	0	0
E-102	9	5,000+	0	0	0	0
	16	1,280	0	0	0	0

TABLE II.
Rate of Destruction at 61.5°C of Hemagglutinin of the Amdall Strain* of Type B Influenza Virus in Allantoic Fluid Containing Different Concentrations of Formalin.

Time at 61.5°C (hr)	Concentrations of formalin, %						
	0	0.01	0.025	0.05	0.10	0.25	0.50
0	5000	5000	5000	5000	5000	5000	5000
.25	0	640+	2560	5000	5000	1280+	0
.5	0	320	1280+	2560+	5000	160	0
1	0	80	640	1280+	1280+	0	0
2	0	0	320	1280	1280	0	0

* In sixth egg-passage.

tions of formalin on one of the recently isolated Type B strains and Table III shows the effect of 0.05% formalin on several other strains as well. In terms of the effect of formalin in a concentration of 0.05%, it is seen that variations exist among the strains shown in Table III, all of which had been isolated from the same outbreak. It appears from these data that the Lee strain is exceptional among the Type B strains, thus far tested, with respect to the effect of the presence of formalin on the stability of the hemagglutinating component of the virus. In view of the variation in heat-stability of 3 different passage lines of the Lee strain reported previously,[1] it was of interest to find that all 3 lines behaved in the same exceptional way in the presence of formalin.

Table IV illustrates the observations made in tests of 3 Type A strains isolated in this laboratory, other than PR8 and Weiss. All 3 showed a slight enhancement of stability in the presence of formalin, but the effect was strikingly less marked than in the case of the Type B strains shown in Tables II and III. Moreover, a somewhat higher concentration of formalin was required to produce the slight effect evident in the Type A strains shown in Table IV.

In summary, these data show that in the

TABLE III.

Rate of Destruction of Hemagglutinin of 5 Strains of Type B Influenza Virus from the 1945-46 Epidemic. Comparison of Formalinized and Unformalinized Allantoic Fluids.

Time at 61.5°C (hr)	Amdall		Neubert		Sadowski		Mindell		Baker	
	No Formal.	.05% Formal.	No Formal.	.05% Formal.	No Formal.	.05% Formal.	No Formal.	.05% Formal.	No Formal.	.05% Formal.
0	2560+	2560+	2560+	2560+	2560+	2560+	1280+	1280+	1280+	1280+
.25	0	—	0	—	0	—	0	—	*0	*160+
1	0	2560+	0	1280	0	640+	0	1280	*0	*40
2	0	2560	0	640	0	320	0	640+	*0	*0
4	0	1280	0	320	0	20+	0	320		
6	0	320	0	80	0	0	0	80		
8	0	20+	0	0	0	0	0	0		
10	0	0	0	0	0	0	0	0		

* Successive intervals were 5, 10, 15 minutes instead of time shown in first column.

TABLE IV.

Rate of Destruction at 61.5°C of Hemagglutinin of 3 Strains of Type A Influenza Virus in Allantoic Fluids Containing Different Concentrations of Formalin.

Strain and passage	Time at 61.5°C (min)	Concentrations of formalin, %						
		0	.01	.025	.05	.10	.25	.50
Johnson	0	5000+	5000+	5000+	5000+	5000+	5000+	5000+
(1943)	15	20	20	40+	160+	160+	640+	40+
Type A	30	0	0	0	20+	160	320	0
F=0	60	0	0	0	0	20+	20+	0
M=0	120	0	0	0	0	0	0	0
E=9								
Scott	0	2560+	2560+	2560+	2560+	2560+	2560+	2560+
(1943)	5	0	0	0	20	40+	0	0
F=0	15	0	0	0	0	0	0	0
M=0								
E=7								
Baum	0	10,000	10,000	10,000	10,000	10,000	5000+	0
(1941)	5	640+	640+	2,560	2,560+	2,560+	0	0
Type A	15	0	0	0	40+	320+	0	0
F=1	30	0	0	0	0	0	0	0
M=10								
E=11								

presence of small concentrations of formalin the hemagglutinin of all but one of 19 strains of influenza virus tested is more stable when subjected to a temperature of 61.5°C and that the effect on different strains varies.

In an effort to determine the influence of time on the reaction between formalin and the hemagglutinating factor tests were made of heat stability at intervals after storage at 4°C. In mixtures of formalin and the Weiss or PR8 strains tested immediately after preparation or after storage for 2 days or 2 weeks, the inactivation curves were indistinguishable, provided the concentrations of formalin present were not great enough to cause destruction of hemagglutinin at the 4° storage temperature. In another experiment, in which formalin was added *after* the virus preparation had been heated for different periods of time, the hemagglutinating capacity was not restored; although in an aliquot the hemagglutinating capacity was retained when formalin had been added *prior* to heating for corresponding intervals. It would seem, therefore, that the presence of formalin delays the destructive effect of heat on hemagglutinin; however, the addition of formalin to a heated virus suspension does not reactivate the hemagglutinin.

Discussion. The observations reported in this communication are of interest with respect to several questions.

It has been noted with almost all strains of influenza virus examined that the heat-stability of the hemagglutinating property was enhanced in the presence of low concentrations of formalin. This is similar to the finding of Schmidt, Glenny, Ramon, and others who reported that the stability of diphtheria toxin to heat-denaturation was increased after treatment with formaldehyde for production of toxoid.[7] It is also known that formalinized pneumococci are more stable than unformalinized organisms[8] and that greater stability to the denaturing action of heat can be produced by formaldehyde treatment of proteins.[7] The exact mechanism of the formaldehyde effect, in which toxicity or infectivity is destroyed without impairing other antigenic properties, is unknown. Many hypotheses have been suggested.[7,9,10] The present observations support the speculation that in addition to blocking or destroying the chemical groupings necessary for toxicity or infectivity, some intramolecular rearrangement occurs producing an effect that might be described as "fixation" of other reactive groups.

A curious fact with respect to influenza virus is the variation observed in the behavior of the hemagglutinating factor of different strains in the presence of formalin. Lack of uniformity in the reaction of the various strains of virus with so simple a substance as formaldehyde suggests that the chemical groupings in the constitution of the individual strains differ widely, either quantitatively or qualitatively.

It is of further interest that differences in the formalin effect were observed among strains of virus isolated from different patients in the same geographical area during a recent epidemic of influenza B.[6] These same strains have been found to possess differences in serological[6] and physical[1] properties. Variations in virulence and serological differences among strains of influenza virus have been described previously[11] and the problems posed by strain variations have been discussed by Francis,[12] Andrewes,[13] Burnet,[14] and others. The fact that strains of virus from the same outbreak are intrinsically different indicates that variants manifested by differences in certain properties do occur very readily in nature. An agent, so labile in its genetic constitution as to vary in the course of multiplication in the same host or from host to host, may be expected to alter its biological behavior quite unpredictably. This characteristic may serve to explain, in part, the striking variability of influenza virus activity both clinically and epidemiologically.[15]

With respect to the more immediately practical question of influenza virus vaccines, the finding of an increased stability of the virus hemagglutinin after formalinization of all but one of the strains tested suggests that the problem of a useful inactivating agent had been solved. The exact relationship between hemagglutinating activity and immunizing activity is still under investigation. The trend of studies reported[16,17] as well as those in progress invites the speculation that the technics employed may furnish information of value with respect to the relative stability of the immunizing capacity of influenza virus vaccines prepared or treated in various ways.

Summary. The observation of an increase in heat-stability of the hemagglutinating property of influenza virus in the presence of low concentrations of formalin has been described. Differences in the effect of formalin on different strains has been reported and discussed.

The technical assistance of Miss Lee Whyte is gratefully acknowledged.

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15526 P

Bilirubin, Bromsulfalein, Bile Acids, Alkaline Phosphatase and Cholesterol of Thoracic Duct Lymph in Experimental Regurgitation Jaundice.

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Saunders[1] was the first to ligate the hepatic duct in dogs in 1795. Since then many others have attempted to elucidate the problem of regurgitation jaundice. An outstanding contribution and review on the subject is given by Mayo and Greene.[2]

The purpose of the present study was to determine the content of bilirubin, bile acids, alkaline phosphatase, and cholesterol, and of bromsulfalein following its administration intravenously, in the plasma and thoracic duct lymph of dogs, after ligation of the common bile duct.

Methods and Materials. Five male dogs were used ranging from 10 to 15 kg. Thirty to 40 mg of pentobarbital sodium per kg of body weight were used as anesthesia. The thoracic duct was cannulated at its entrance in the left jugular vein. Three drops of 25% sodium citrate solution were added to 10 cc of blood or lymph, to prevent coagulation, all determinations being made on plasma. Biliary obstruction was produced by a double ligation and severance of the common bile duct with previous ligation of the cystic duct. Bilirubin was determined by a modification[3,4] of Malloy and Evelyn's method.[5] Bromsulfalein was administered in the right external jugular vein in a dose of 2 mg per kg of body weight. The concentration of dye was then determined in the following manner:

Three cc of triethyl phosphate (Eastman Kodak Company) was added to 1 cc of plasma or lymph in a centrifuge tube. This was shaken thoroughly and centrifuged. Two cc of the supernatant fluid were then added to 8 cc of distilled water in an Evelyn tube. A blank reading was made in the Evelyn photoelectric colorimeter, using a 580 filter. Two drops of 20% KOH were then added to develop the color, and a reading was again taken, after which the concentration was de-

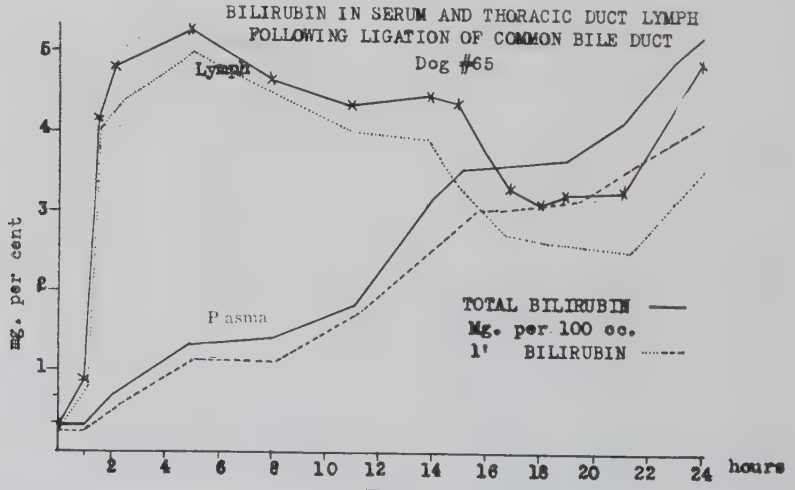

Fig. 1.

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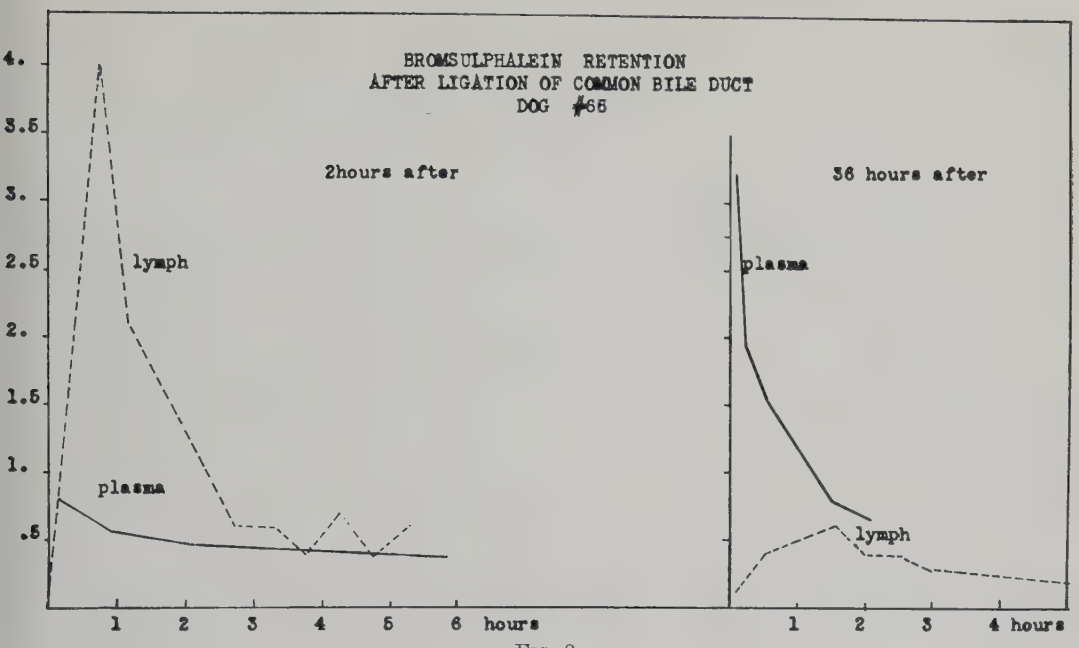

FIG. 2.

termined from a curve of the pure dye in known concentration. Bile acids were determined as cholic acid by Josephson's method.[6] Cholesterol was determined by the Sperry Schoenheimer method.[7] The alkaline phosphatase was determined by the method of King and Armstrong.[8]

Results. As seen in Fig. 1, bilirubin appears promptly in the thoracic duct lymph, being detectable in the blood a few hours later. Most of the bilirubin was of 1′ or prompt reacting type[3,4] in both lymph and plasma.

The data in Fig. 2 relates to injection of bromsulfalein at 2 hours and at 36 hours following the ligation of the common bile duct. In the early period of the obstruction, it is seen that the dye disappears promptly from the blood to be regurgitated at high concentration into the thoracic duct lymph. In the later period of obstruction, however, the dye is retained in the blood for several hours and little or none appears in the thoracic duct lymph.

Fig. 3 represents a typical experiment as regards bile acids in the thoracic duct lymph and blood, following ligation of the common bile duct.

These results are in accord with previous studies in which the Pettenkofer test was used, as discussed by Mayo and Greene.[2]

Typical data for the cholesterol in lymph and blood, in these experiments, is shown in Fig. 4. It is seen that the concentration of the total plasma cholesterol remained about the same during the 24 hours of observation. From this, it appears that if regurgitation of cholesterol from bile to lymph and thence to blood, does occur, it is considerably slower than that of the bile acids and the prompt reacting bilirubin.

Fig. 5 shows the rise in alkaline phosphatase in the blood and lymph. It is of considerable interest that distinct increases in phosphatase activity in thoracic duct lymph were observed.

Summary. The present results support the belief that, following common duct ligation, bile "regurgitates" into the lymph, and thence, via the thoracic duct into the blood. The appearance of bile acids and bilirubin in the thoracic duct lymph, as previously reported, is confirmed. The present study also reveals

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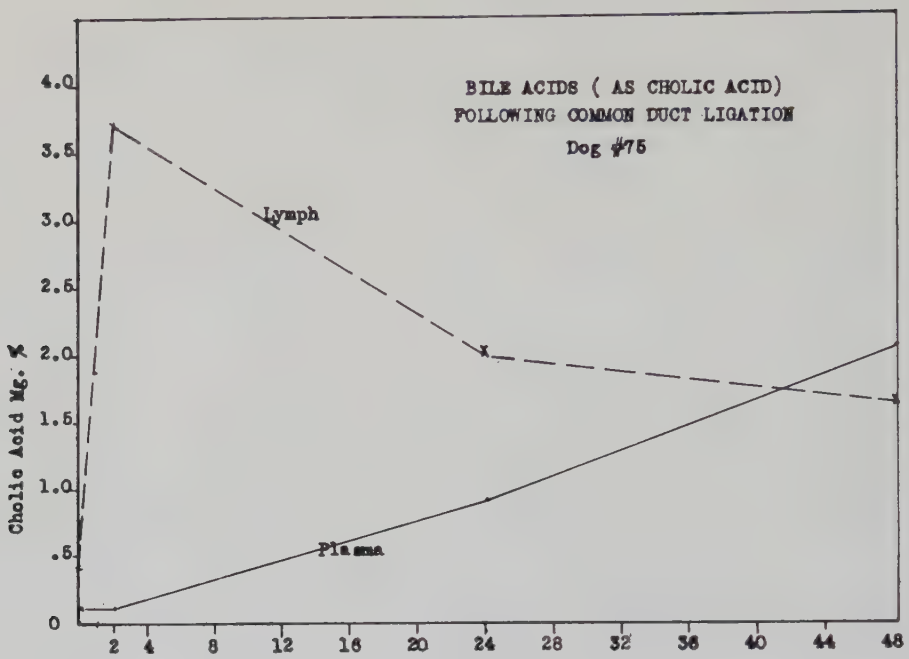

FIG. 3.

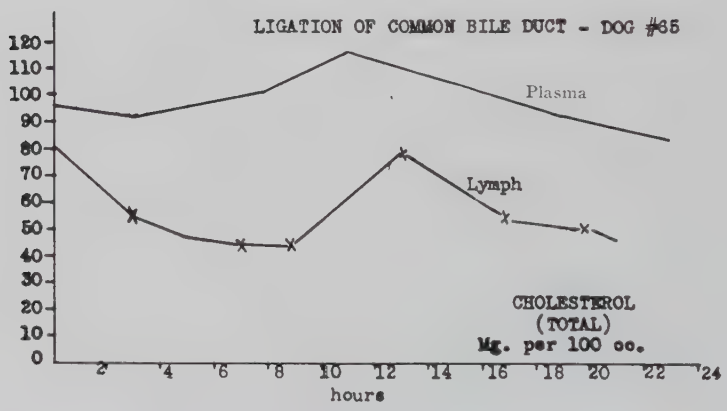

FIG. 4.

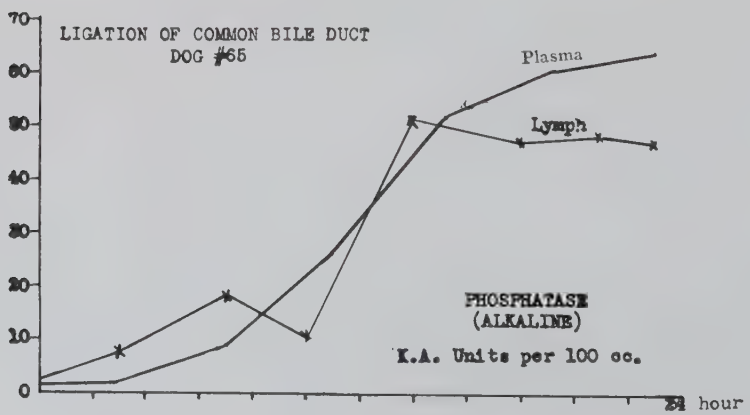

FIG. 5.

that the bilirubin of the lymph, under these circumstances, is mainly of the prompt reacting type, a finding in accord with the concept of a regurgitation of bile into the lymph.

During the early phase of biliary obstruction, the injected bromsulfalein quickly appeared in the lymph. After 36 hours, however, it was not removed from the blood and did not appear in the lymph in appreciable amount.

The cholesterol, on the contrary, did not increase significantly in the lymph within 24 hours, while the alkaline phosphatase behaved in an intermediary manner.

The writer wishes to acknowledge his indebtedness to Dr. C. J. Watson for helpful criticism and advice with relation to these studies.

15527

Effect of Anoxic Anoxia on Gastric Emptying Time of Rats Fed Corn Oil.

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Anoxic anoxia has been shown to cause a delay in the emptying time of the stomach in the dog and in man. Moreover, in man, as in the case of the dog, the more severe the degree of anoxia, the greater is the delay in gastric emptying.[1]

Studies on the effect of this type of anoxia on the absorption of fat from the alimentary tract in rats have shown that the amount of fat absorbed by animals subjected to partial pressures of oxygen of 63 mm and 53 mm Hg was significantly less than for control animals; the difference was greater for the greater degree of anoxia.[2] That these findings cannot be explained on the basis of a prolonged emptying time of the stomach, however, is shown by the following experimental results.

Using the technic previously described,[2] adult albino rats of both sexes, weighing between 200-300 g were given 1.5 cc corn oil (1.385 ± 0.01 g) and subjected to a partial pressure of oxygen of 53 mm Hg (7.03% oxygen) for 2-, 3- and 4-hour periods. Simultaneously-fed control animals were kept at atmospheric pressure. The amount of fat remaining in the stomach was quantitatively determined and calculated as per cent of the amount fed. Preliminary experiments on rats killed immediately after feeding showed that the mean percentage recovery of the amount of fat administered was 96.5 (range 92.3 to 99).

The data, Table I, show that the amount of

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TABLE I.
Effect of Anoxic Anoxia on Gastric Emptying Time of Rats Fed Corn Oil.
Oxygen tension—53 mm Hg (7.03% oxygen).

Absorption time	2 hr		3 hr		4 hr	
	No. of rats	Fat in stomach* %	No. of rats	Fat in stomach %	No. of rats	Fat in stomach %
Controls	12	68.4	11	49.5	12	41.3
Anoxic	11	37.4	12	30.0	12	35.7
St. Dev.		17.0		16.5		13.4
P. (Fisher's)		<0.01		0.01		>0.3

* Expressed as per cent of the amount of fat fed.

fat remaining in the stomachs of the anoxic rats 2 hours and 3 hours after feeding was significantly less than for the corresponding controls. On the other hand, no statistically significant difference was found at the end of 4 hours. (The individual variation which was greater for the rats subjected to anoxia, than for the controls, accounts for the apparently larger amount of fat in the stomach after 4 hours absorption time than after 3 hours). Although a delay in gastric emptying may have occurred later than 4 hours, these findings indicate an initial acceleration of the emptying of the stomach of rats exposed to diminished oxygen tension. For the purpose of these experiments, the results obtained show that the decreased rate of absorption of fat in rats subjected to anoxic anoxia, as previously reported, cannot be explained on the basis of a prolonged gastric emptying time.

Schnedorf and Orr[3] reported that increasing degrees of anoxemia produced by inhalation of 15, 10 and 5% oxygen in nitrogen resulted in a marked and progressive decrease below normal in the flow of bile in nembutalized dogs. The decreased rate of fat absorption observed in rats subjected to partial pressures of oxygen of 63 mm and 53 mm Hg (8.35 and 7.03% oxygen, respectively) might reasonably result from a diminished flow of bile. Work on this phase of the problem is in progress.

Summary. Adult albino rats fed corn oil showed an initial acceleration of the emptying of the stomach on exposure to diminished oxygen tension. A decreased rate of absorption of fat in rats subjected to anoxic anoxia cannot be explained on the basis of a prolonged gastric emptying time.

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15528

Latent Period Between Electrical and Pressure Pulse Waves Corresponding to Right Auricular Systole.*

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The exact time relationship between the onset of electrical activity and the appearance of a pressure wave corresponding to auricular systole has been, heretofore, unknown in man. The development of the technic of catheterization of the right heart[1-4] and the use of a multiple blood pressure recorder with a synchronous electrocardiogram has made possible the study of this time relationship.

Preliminary tests for measuring the difference of transmission time of electrical impulses as recorded with the ECG and simultaneous mechanical impulses from the tip of a catheter connected with a Hamilton manome-

* Under grants from the Commonwealth Fund and the Life Insurance Medical Research Fund Gift for Study of Action of Certain Cardiovascular Drugs.

† Captain Medical Corps Reserve, U. S. Army, on detached service from Aero-Medical Laboratory, Wright Field, Dayton, Ohio.

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4 Cournand, A., Bloomfield, R. A., and Lauson, H. D., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 73.

TABLE I. Time Relationship of Electrical and Pressure Pulse Waves Corresponding to Auricular Systole in Man.

Case	Date	Clinical diagnosis	Age, yrs	Heart rate	Avg time in sec.*			
					Beginning of P wave to beginning of auricular systole	Peak of P wave to peak of auricular systole	Beginning of P wave to peak of auricular systole	P-R interval of E(G
			8 Cases with Essentially Normal Cardiac Function.					
L.B.	1/29/46	Pulmonary fibrosis	50	73.2	—	.12	.16	.16
B.O.	2/19	Essentially normal	27	78.2	—	.095	.15	.15
E.H.	2/28	"	23	85.2	.11	.11	.16	.16
E.H.	3/12		23	85.6	.11	.11	.16	.16
E.L.	3/5	Art. pneumothorax	27	90.8	.095	.095	.17	.19
J.L.	3/26	6 yrs post-pneumonectomy	18	72.0	.12	.12	.18	.17
E.C.	4/15	Essentially normal	31	81.2	.11	.11	.14	.17
A.S.	4/17	Art. pneumothorax	22	111.5	—	.13	.16	.15
Avg				85.0	.11	.11	.160	.164
			2 Cases with Cardiac Abnormalities.					
G.L.	3/19	Tetralogy of Fallot	8	113.0	.055	.055	.125	.13
R.B.	3/28	Scleroderma	59	83.0	.17	.17	.20	.25

* Corrected for .01 sec difference between electrical and mechanical conduction time.

Fig. 1.
A. Mechanical conduction.
B. Electrical conduction.
Transmission time difference of simultaneously-induced electrical and mechanical impulses. (See text for description).

ter were made in the following manner; one electrode of the ECG was attached to a small rubber balloon, fastened on the distal end of a No. 8 cardiac catheter, with the proximal end connected by lead tubing to a Hamilton manometer. The entire mechanical system was filled with sodium citrate with all air bubbles removed. Tapping the attached electrode with another electrode (circuit of lead II used), initiated simultaneously an electrical and mechanical impulse that was transmitted to the string of the ECG and the manometer membrane; the resulting deflections were photographed using a camera speed of 50 mm per second. As illustrated on Fig. 1, the time interval between the beginning of the electrical and the mechanical deflection is approximately 0.01 second.

The data obtained on 10 human subjects are tabulated in Table I, and representative tracings shown on Fig. 2. In 8 individuals with essentially normal cardiac function the latent period of the right auricle measured from the time of the beginning of the P wave to the beginning of the auricular systolic pulse wave averaged 0.11 second after the 0.01

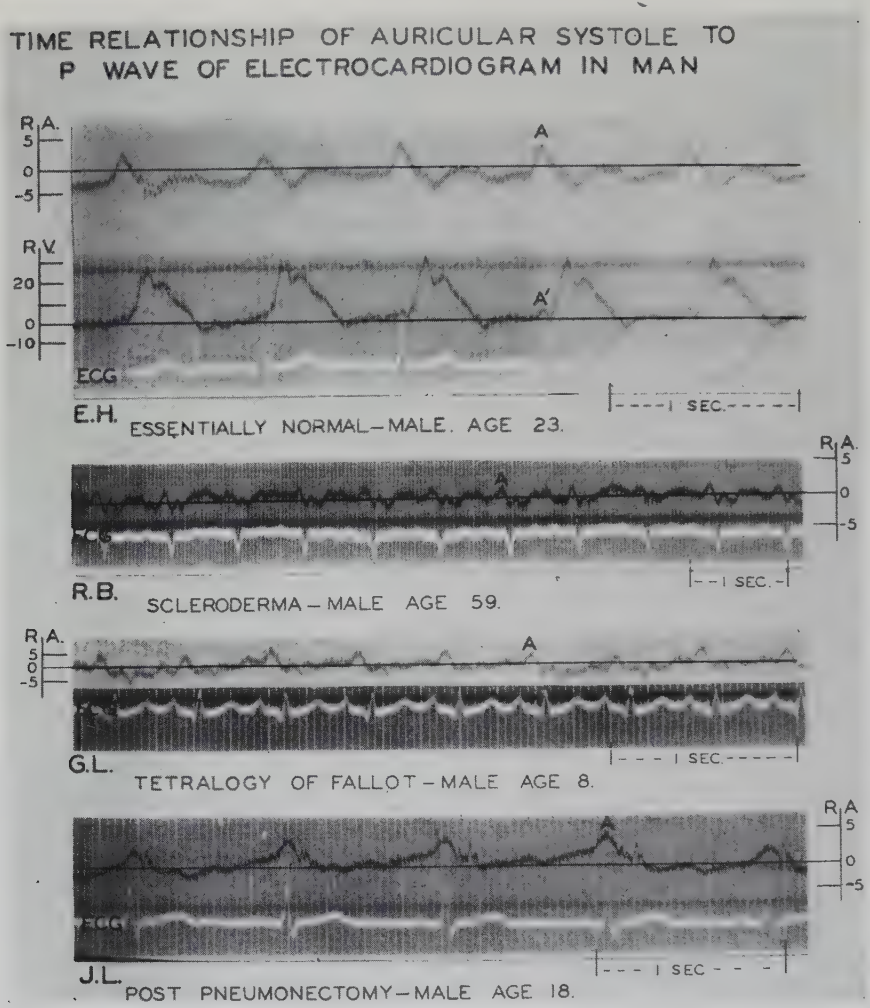

Fig. 2.

A. = auricular systole.
A'. = auricular systole on right ventricle tracing.
R.A. = right auricle. R.V. = right ventricle.
E.C.G. = electrocardiogram—lead II.
All pressures in mm mercury.
Time interval between electrical and pressure pulse wave corresponding to the auricular systole in normal man and in 2 cases with cardiac abnormalities.

second correction was subtracted for the difference between the electrical and mechanical conduction. In 3 cases it was necessary to take the measurements from the peak of both electrical and pressure waves as the exact beginning of the auricular systole was not sharply defined on the tracing. In all cases where both determinations could be made the time lag was the same as seen in Table I. The average time from the beginning of the P wave to the peak of the auricular systolic pulse wave was 0.16 second, ranging from 0.14 sec. to 0.18 sec. The P-R interval averaged 0.164 sec. (range 0.15 sec. to 0.19 sec.)

The results in 2 cases of cardiac abnormality are shown in Table I and the tracings illustrated in Fig. 2. In the case of an 8-year-old child with Tetralogy of Fallot, the time lag between the electrical and pulse wave was 0.055 sec. The heart rate was rapid, 113 per minute. The time from the beginning

of the P wave to the peak of the auricular systole was shorter than the normal limits calculated above. In one case of a 59-year-old male diagnosed as scleroderma with involvement of the lungs, marked pulmonary hypertension and evidence of partial heart block, the latent period was 0.17 sec. The time from the beginning of the P wave to the peak of the auricular systole was 0.20 sec. in this case. This may represent some delay in the spread of the sinus impulse through the right auricle.

Summary. 1. In 8 adult subjects with essentially normal hearts the latent period between the beginning of the electrical P wave and pressure pulse wave corresponding to the right auricular systole averaged 0.11 sec. 2. In one case of a child with a rapid pulse and Tetralogy of Fallot the latent period was much shorter, 0.055 sec. 3. In one case of scleroderma with pulmonary hypertension and partial heart block the latent period was prolonged to 0.17 sec.

15529 P

Influence of Thiourea on Development of the Chick Embryo.

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While investigating the effect of several substances which may influence the growth of rickettsiae in the yolk sac of the chick embryo, some effects on the embryo itself have been observed, and these have been followed up for thiourea.

Thiourea and related substances have been shown to affect the thyroid function in adult animals, *viz.*, rat,[1,2] mouse and dog,[1] rabbit,[3] and chick.[4] Thiourea has also been found to inhibit metamorphosis in tadpoles[5–7] and cleavage of sea urchin eggs.[8]

Thiourea is introduced into the yolk sac of Leghorn embryos according to the technic described by Cox.[9] Aseptic precautions are strictly adhered to throughout the whole procedure. Fertile eggs incubated at 39°C for 7-17 days are used. The shell covering the air sac is washed with 5% phenol and the injections are made into the yolk through a needle-sized opening in the air sac by means of a hypodermic syringe and a 22-gauge needle. The hole is then sealed with paraffin and the eggs are returned to the incubator. Amounts of thiourea used were 0.3 to 3 mg per egg. In order to avoid mechanical damage to the embryo, the fluid introduced should not exceed 0.5 ml; a similar volume of distilled water serves as control. Eggs were daily inspected by candling and dead ones were discarded.

Results. While the controls hatched after 21 days (on very few occasions after 20 or 22 days), hatching of embryos treated with thiourea was retarded up to 10 additional days. The retardation seemed to depend on 2 factors, (a) age of the embryo at the time of injection; (b) concentration of thiourea. For example, 1 mg thiourea delayed hatching by only 1 day in 17-day egg embryos, while the same amount applied to younger

* The author is indebted to Dr. M. Aschner for his valuable suggestions.

1 Mackenzie, C. G., and Mackenzie, I. B., *Endocrin.*, 1943, **32**, 185.

2 Astwood, E. B., Sullivan, J., Bissel, A., and Tyslovitz, R., *Endocrin.*, 1943, **32**, 210.

3 Bauman, I., Metzger, N., and Marine, D., *Endocrin.*, 1944, **34**, 44.

4 Mixner, J. P., Reineke, E. P., and Turner, C. W., *Endocrin.*, 1944, **34**, 169.

5 Gordon, A. S., Goldsmith, E. D., and Charipper, H. A., *Nature*, 1943, **152**, 504.

6 Gordon, A. S., Goldsmith, E. D., and Charipper, H. A., *Growth*, 1945, **9**, 19.

7 Hughes, A. M., and Astwood, E. B., *Endocrin.*, 1944, **34**, 138.

8 Bevelander, G., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 268.

9 Cox, H. R., *Science*, 1941, **94**, 399.

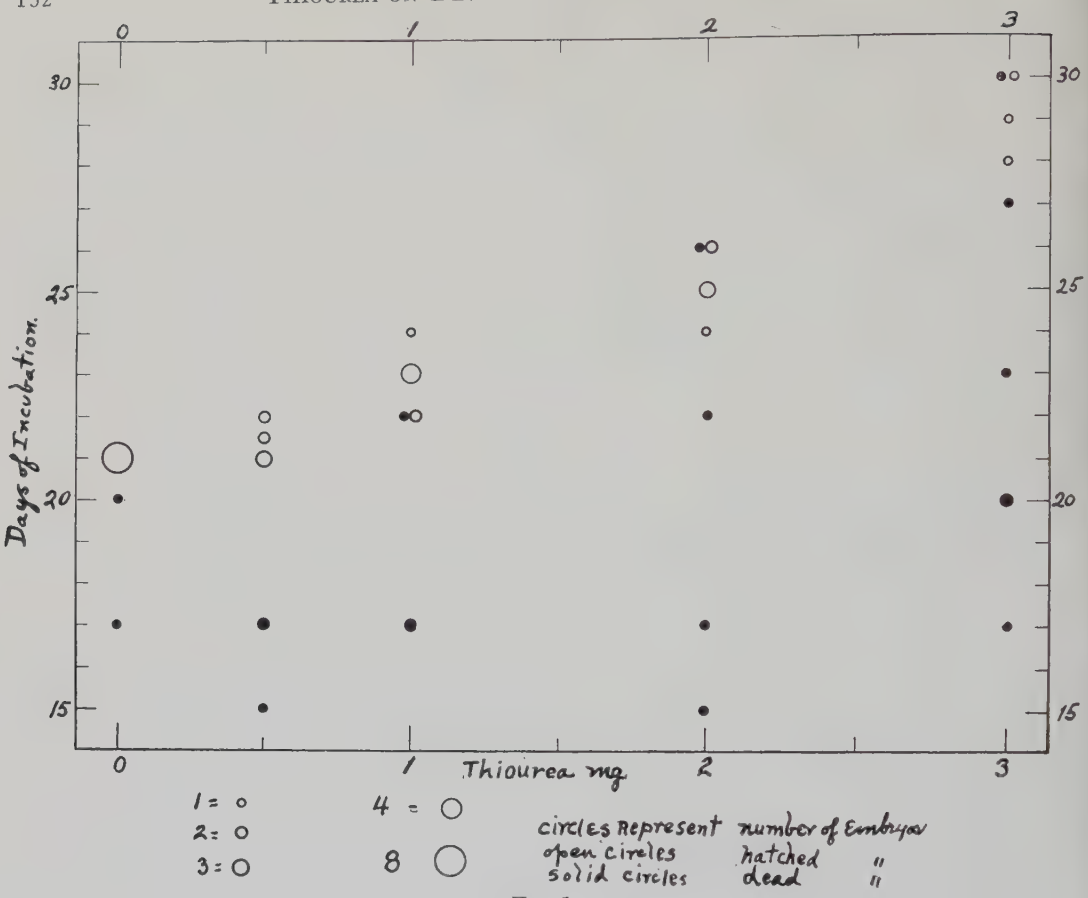

FIG. 1.

Delay in hatching time of Leghorn egg embryos after injection of thiourea at the 14th day of incubation.

embryos (10 days old) caused a delay of 3 days. Two mg of thiourea retarded hatching by 3-7 days, the effect again depending on age. Though hatching time can be extended up to the 30th day by still higher amounts of thiourea (3 mg), hatchability is much impaired being equivalent to only about 5-10% of the controls. In addition, the delay in hatching is associated with other signs of inhibition. Whereas in normal embryos the yolk sac begins to retract on the 19th day, and by the 20th day has entirely entered the abdomen, the yolk sac is not much reduced in size at this stage in eggs treated with thiourea; it can still be found on the 30th day of incubation in those embryos previously treated with a dose of 3 mg thiourea. While in the controls the "egg tooth" enters the air-chamber on the 20th day, treatment with 2 mg thiourea at age of 10-14 days delays this phenomenon by 2-5 days. The results of a typical experiment are given in Fig. 1.

Experiments were also carried out in order to elucidate the mode of action of thiourea. Gordon *et al.*[6] were able to show that the inhibiting effect of thiourea on tadpoles can be reversed by thyroxine. In our experiments it was difficult to prove the reversing effect of thyroxine as it was found to be very toxic especially for embryos up to 14 days. Thyroxine in nontoxic concentrations had no antagonizing activity at all. However, in some experiments with 17-day-old eggs, 10 μg of thyroxine fully neutralized the action of 2 mg thiourea when the 2 drugs were simultaneously applied (molecular ratio 1:2,000). The experiments suggest that in the chick embryo, as in other animals, the inhibiting effect of thiourea may be due to interference with normal thyroxine metabolism.

15530 P

A New Class of Tuberculostatic Substances.

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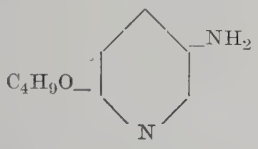
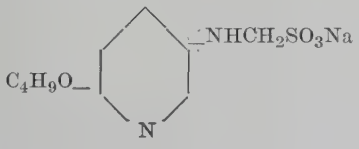
The search for substances having tuberculostatic activity has in recent years engaged increasing interest in many laboratories. Using an *in vitro* technic in which a rapidly growing avirulent strain of "human" tuberculosis organism is subjected to various concentrations of compounds under test makes feasible the "screening" of large numbers of substances in a comparatively short time. This method, the details of which are essentially the same as described earlier,[1] has brought to attention 5-amino-2-butoxypyridine



which was investigated in some detail. Several hundred derivatives of this substance were synthesized† one of which, the sodium formaldehyde bisulfite derivative



relatively among the least toxic,[2] is included in this report.

The growth of 607, the rapidly growing strain of the tubercle bacillus, was inhibited by these compounds in dilutions in excess of 1 to 3 million. Other strains, virulent and recent isolations, were inhibited in dilutions as high as 1 to 100 million. A remarkable feature associated with this high tuberculostatic effect is that while several bacterial genera have been investigated only Mycobacterium was susceptible to the action of these substances in significant dilution. The data presented in Table I are typical of the results obtained in many-time repeated experiments. The specificity for Mycobacterium is illustrated by the lack of inhibition of growth of Staphylococcus, Streptococcus, Pneumococcus, *B. mycoides, E. coli,* and others, even in what may be termed high concentrations. This specificity contrasts sharply with the lack of specificity shown by sulfones and sulfonamides[1] which are active against the rapidly growing avirulent *Mycobacterium tuberculosis 607* (line No. 1) as well as *E. coli* in a synthetic medium (line No. 10). Comparison is also made with promin and its parent substance p,p′-diaminodiphenylsulfone.

It is important also to note that while the more active sulfonamide and sulfones are capable of inhibiting the growth of the avirulent strain of tubercle bacillus in comparatively high dilution (line No. 1), these compounds are relatively ineffective against other more virulent strains (lines 2, 3 and 4).

The antagonistic effect of para-aminobenzoic acid[3] and methionine[4] for sulfonamides is well known. As suspected these substances did not inhibit the activity of I or II, as shown by lines 1, 14 and 15 in Table I. The bacteriostatic activity of our compounds against the tubercle bacillus is likewise not antagonized by the incorporation of adequate quantities of riboflavin, calcium pantothenate, adenine, guanine, thiamine, uracil, nicotinic acid, biotin, culture filtrates from staphylococci, pneumococci and tubercle bacilli, pus from streptococcal lymphadenitis, constituents from beef culture media, peptone (which

* The author wishes to express his grateful acknowledgment of the technical assistance rendered by Miss Anna Kelly and Miss Mary Rothlauf.

1 Fitzgerald, R. J., and Feinstone, W. H., Proc. Soc. Exp. Biol. and Med., 1943, **52**, 27.

† 5-Amino-2-butoxypyridine and its derivative were synthesized by the chemical staff of the Pyridium Corp. under the supervision of Drs. E. T. Tisza and H. L. Friedman.

2 To be reported elsewhere.

3 Woods, D. D., *Brit. J. Exp. Path.*, 1940, **21**, 74.

4 Harris, J. S., and Kohn, H. I., *J. Pharm. and Exp. Therap.*, 1941, **73**, 383.

TABLE I.
Comparative Bacteriostatic Effect of 5-Amino-2-Butoxypyridine and Certain Sulfonamides and Sulfones.

	Organism	Culture medium	Lowest concentration (mg per 100 cc) showing bacteriostasis. Compounds					
			I†	II§	Sulfanil-amide	Sulfa-thiazole	Promin§	p,p′-Diamino-diphenylsulfone‡
1	*M. tbc.* 607‖	Dorset's Synthetic	0.031	0.062	2.0	0.062	4.0	0.062
2	*M. tbc.* H37RV	Proskauer Beck	0.0039	0.0078		0.5	64.0	0.5
3	*M. tbc.* G2¶	,, ,,	0.00099	0.00099	64.0	2.0	16.0	2.0
4	*M. tbc.* Benton**	,, ,,	0.0019	0.00099	32.0	0.5	16.0	2.0
5	*M. tbc.* bovine‖	,, ,,	0.0078	0.015				
6	*M. avium*‖	,, ,,	0.156	0.0312				
7	*M. phlei*‖	,, ,,	0.0039	0.0039				
8	*M. stercoris*‖	,, ,,	0.0039	0.015				
9	*M. lepræ*‖	,, ,,	0.031	0.031				
10	*E. coli*	Sayhun Synthetic	64.0	64.0	2.0	0.031	10.0	1.0
11	*Strep. hem.* C203	Beef Infusion Broth	32.0	64.0	32.0	16.0		10.0
12	*B. mycoides*	Peptone Dextrose Broth	8.0	32.0		8.0		
13	Pneumococcus SV1	,, ,, ,,	64.0	64.0	8.0	0.5		
14	*M. tbc.* 607	Dorset's + 0.1 mg % para-aminobenzoic acid	0.031	0.062	64.0	8.0		8.0
15	*M. tbc.* 607	Dorset's + 1.0 mg % methionine	0.031	0.062	16.0	0.25		1.0

* Complete or almost complete visible inhibition of growth for 72 hours following inoculum or in the case of slow growing tubercle bacillus for 30 days. Tubercle bacilli inoculum either by small pellicle or suspension of about 20,000 organisms per cc.

† I—5-Amino-2-butoxypyridine as the hydrochloride.

‡ II—Sodium formaldehyde bisulfite of 5-amino-2-butoxypyridine.

§ Promine and p,p′-diaminodiphenylsulfone supplied through the kindness of Dr. L. A. Sweet, Parke, Davis & Co.

‖ Obtained from The American Type Culture Collectioon. *M. lepræ* is of course of questionable identity.

¶ Obtained through the courtesy of Dr. Guy P. Youmans, Northwestern University Medical School.

** Obtained through the courtesy of Dr. William H. Feldman, Mayo Foundation.

antagonizes sulfonamide activity[5] and whole blood and serum up to 25%.

The antimycobacterial activity of 5-amino-2-butoxypyridine and its derivative is primarily bacteriostatic rather than bactericidal. (We hold the term bactericidal in its strictest sense, *i.e.*, complete sterilization of a sizable inoculum in a culture medium which, when free of inhibitory compounds, supports vigorous growth). Concentrations of 5-amino-2-butoxypyridine 1,000 times the minimum tuberculostatic concentration do not consistently render the system free of viable organisms.

The fact that these compounds are bacteriostatic rather than bactericidal and strikingly specific for the acid-fast group of organism suggest a mechanism of action involving the interference with some essential metabolic process common to species of Mycobacterium but which is lacking, has readily available alternatives or is nonessential in other genera of organisms.

5 Lockwood, J. S., and Lynch, H. M., *J. A. M. A.*, 1940, **114**, 935.

15531

Bilateral Nephrectomy in Rats: Blood Chemistry, Longevity and the Effect of Aluminum Hydroxide.

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In the hypocalcemic tetany of the uremic patient aluminum hydroxide reduces the tendency towards convulsions by elevating the serum calcium[1] presumably by preventing the absorption of the phosphate ion.[2] Subsequently, these studies were initiated in order to observe the effect of aluminum hydroxide gel on the survival time and on the blood levels of sulphates, phosphates, calcium, urea and cholesterol in rats following bilateral nephrectomy.

Method and Material. A total of 108 rats were used in this study. Fifty-one rats were employed in the longevity study of which 26 (16 males and 10 females) served as controls and 25 (15 males and 10 females) as experimental animals. The males weighed 400 to 500 g and the female rats 250 to 300 g. Both kidneys were removed by a transperitoneal approach through a midline ventral incision under ether anaesthesia. Sterile technic was utilized throughout. Each of the animals in the experimental group was given 5 ml of 4% aluminum hydroxide gel by stomach tube. An analgesic dose of ether was administered prior to the passage of the stomach tube in order to permit intubation without the use of a gag. The aluminum hydroxide was given once daily for 2 days prior to nephrectomy, then 4 to 6 hours after surgery, and thereafter once every 24 hours until the animals expired. The survival time of these rats was compared with the control group.

For the study of variations in blood chemistry, 28 non-nephrectomized male rats were anaesthetized with ether after which the abdominal aorta was exposed through a midline ventral incision. Blood was then aspirated from the aorta by means of a 26-gauge needle and a 2-ml hypodermic syringe. Seventeen male rats were nephrectomized and 48 to 50 hours postoperatively blood was withdrawn from the abdominal aorta as described above. In addition, 11 male rats were nephrectomized but were given aluminum hydroxide by stomach tube. These animals were also sac-

* Research Assistant in Medicine, New York Post-Graduate Medical School, Columbia University, New York City.

1 Personal observation, unpublished data.

2 Evans, G. T., and Flink, E. B., *Modern Med.*, 1945, **13**, 63; Fauley, G. B., Freeman, S., Ivy, A. C., Atkinson, A. J., and Wigodsky, H. S., *Arch. Int. Med.*, 1941, **67**, 563.

rificed 48 to 50 hours postoperatively in order to obtain blood from the abdominal aorta. The bloods were analyzed for serum inorganic sulphates, serum inorganic phosphates, serum calcium, whole blood urea and whole blood cholesterol.

Serum calcium was determined by the Clark-Collip[3] modification of the Kramer-Tisdall method and serum inorganic phosphates by the Lowenberg and Mattice[4] modification of the Benedict-Theis[5] method. Blood serum was analyzed for inorganic sulphates according to the method of Power and Wakefield.[6] The procedure of Myers[7] was used for the estimation of whole blood urea nitrogen. The whole blood cholesterol was determined by the method of Sackett.[8] Since the amount of blood withdrawn from the abdominal aorta of any one rat was frequently insufficient for complete studies, several samples were mixed thoroughly by gentle shaking and an aliquot portion of this pooled blood was used.

Results. Of 26 control rats, 14 or 54% expired on the 3rd day following bilateral nephrectomy while 12 (46%) died on the 4th day. The sex of the animal was not an influencing factor. In 25 nephrectomized rats receiving aluminum hydroxide, 12 (48%) expired on the 3rd day, 7 or 28% on the 4th day, while 20% lived beyond the control survival time (4 for 5 days and 1 for 6 days).

The average whole blood urea nitrogen of 28 normal rats was found to be 17.3 mg % (range 14-20.5), average serum calcium 9.5 mg % (range 8.4-10.4), average serum inorganic phosphates 6.9 mg % (range 4.5-9.2), average serum inorganic sulphates 2.97 mg % (range 2.70-3.49) and whole blood cholesterol 55.5 mg % (range 42-70). The 17 nephrectomized rats which did not receive aluminum hydroxide had an average urea nitrogen of 194 mg % (range 130-250), calcium 10.4 mg % (range 9.4-12.0), phosphates 8.1 mg % (range 6.5-10.8), sulphates 23.2 mg % (range 15-28.8) and cholesterol 70.8 mg % (range 60-96) 48 to 50 hours postoperatively. Bilateral nephrectomy, therefore, caused a marked retention of urea nitrogen and sulphate and a somewhat smaller rise in calcium, phosphate and cholesterol. The 11 nephrectomized rats receiving aluminum hydroxide showed an average urea nitrogen of 182 mg % (range 150-203), calcium 9.5 mg % (range 8.2-9.8), phosphates 7.9 mg % (range 6.1-9.4), sulphates 21.6 mg % (range 14.1-26.3) and cholesterol 65.5 mg % (range 60-69) 48 to 50 hours postoperatively. Apparently, the administration of aluminum hydroxide to bilateral nephrectomized rats was accompanied by slight decreases in each of the chemical constituents studied.

Discussion. The survival time of bilateral nephrectomized rats in this series is consistent with that of other investigations.[9] The increase of 20% in the longevity rate by the administration of aluminum hydroxide is difficult to explain, although it is conceivable that aluminum hydroxide may adsorb some of the toxic metabolites excreted into the intestinal tract (Adams).[10] It is universally appreciated that a moderate or severe acidosis develops in retention uremia;[11] the retention of the acid sulphate ion is probably the most important factor in the production of the acidosis. Aluminum hydroxide does not influence the acidosis directly since it is insoluble and nonabsorbable.[12] One may postulate that this aluminum compound interrupts, temporarily, the vicious cycle which might exist in nephritic acidosis, namely, the continued resecretion of high concentrations of

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[4] Lowenberg, C., and Mattice, M. R., *J. Lab. and Clin. Med.*, 1930, **15**, 598.

[5] Benedict, S. R., and Theis, R. C., *J. Biol. Chem.*, 1924, **61**, 63.

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[7] Myers, V. C., *Practical Chemical Analysis of Blood*, St. Louis, C. V. Mosby, 1924, p. 45.

[8] Sackett, G. E., *J. Biol. Chem.*, 1925, **64**, 203.

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[10] Adams, W. L., *Arch. Int. Med.*, 1939, **63**, 1030.

[11] Marriott, W. M., and Howland, J., *Arch. Int. Med.*, 1916, **18**, 708; Holt, L. E., and McIntosh, R., *Holt's Diseases of Infancy and Childhood*, 10th ed., D. Appleton-Century, 1934.

[12] Report Council on Pharmacy and Chemistry, *J. A. M. A.*, 1941, **117**, 1356.

acid into the stomach, by adsorbing total and free acid.[2,11,13]

The increase in inorganic acids of the blood is perhaps more important than the retention of nitrogenous products particularly insofar as acid-base balance is concerned. Denis[14] has demonstrated an increase of the blood sulphate level in patients with chronic nephritis and Macy[15] has shown an impaired clearance of sulphates in this disease. The present study shows that sulphate retention is appreciable in the nephrectomized rat.

No significant changes either in phosphates or serum calcium levels were noted in the nephrectomized rat. In the presence of prolonged acidosis and proteinuria (calcium adsorbed to protein) such as is found in chronic nephritics one might expect changes in serum calcium; in this study the element of time was too short and the factor of proteinuria absent.

Conclusions. 1. The longevity of the bilateral nephrectomized rat was 3 to 4 days. 2. The oral administration of aluminum hydroxide increased the survival time of 20% of nephrectomized rats to 5 or 6 days. 3. Bilateral nephrectomy resulted in a marked elevation of urea nitrogen and inorganic sulphate in the blood whereas the retention of inorganic phosphate, calcium and cholesterol was insignificant. 4. The oral administration of aluminum hydroxide in the bilateral nephrectomized rat produced a slight decrease in the degree of retention of each of the various blood constituents studied.

[13] Crohn, B. B., *J. Lab. and Clin. Med.*, 1929, **14**, 610; Collins, E. N., Pritchett, C. P., and Rossmiller, H. R., *J. A. M. A.*, 1941, **116**, 109; Einsel, I. H., Adams, W. L., and Myers, V. C., *Am. J. Digest. Dis. and Nutrition*, 1934, **1**, 513; Rossett, N. E., and Flexner, J., *Ann. Int. Med.*, 1944, **21**, 119.

[14] Denis, W., and Hobson, S., *J. Biol. Chem.*, 1923, **55**, 183.

[15] Macy, J. W., *Arch. Int. Med.*, 1934, **54**, 389.

15532 P

Reproduction in Chickens on Synthetic B-Complex Supplement.

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Reproduction in rats on a diet containing the B-factors in synthetic form was previously reported.[1] However, it was not possible to rear chickens on a diet of this type until synthetic pteroylglutamic acid became available.[2] Day-old New Hampshire chicks were placed on the following diet: glucose (cerelose) 58.5 g, purified casein (Labco) 20 g, gelatin 8 g, calcium gluconate 5 g, cystine 0.4 g, choline chloride 0.2 g, inositol 0.1 g, bone ash 2 g, NaCl 0.6 g, KH_2PO_4 0.45 g, K_2HPO_4 0.6 g, $MgSO_4$ 0.25 g, $MnSO_4,4H_2O$ 0.05 g, ferric citrate 0.05 g, $CuSO_4,5H_2O$ 2 mg, $Al_2(SO_4)_3,18H_2O$ 1.6 mg, zinc acetate 1.4 mg, KI 0.6 mg, cobalt chloride 0.4 mg, nickel chloride 0.2 mg, calcium pantothenate 5 mg, niacinamide 5 mg, riboflavin 1 mg, pyridoxine HCl 1 mg, thiamine HCl 1 mg, *p*-aminobenzoic acid 1 mg, pteroylglutamic acid 0.5 mg, 1-acetoxy-2-methyl-4-naphthyl sodium phosphate 0.5 mg, (dl) biotin .04 mg, to which were added vitamin A 1500 U.S.P. units, vitamin D 200 A.O.A.C. units, mixed tocopherols 34 mg, dissolved in corn oil (Mazola) to a total of 3 g. The birds were kept in metal cages with wire floors. In this diet, the fat-soluble factors were fed in a crude form but the water-soluble vitamins, except inositol, were all synthetic and were fed at levels which appeared to be more than sufficient in each

[1] Jukes, T. H., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 625.

[2] Angier, R. B., Boothe, J. H., Hutchings, B. L., Mowat, J. H., Semb, J., Stokstad, E. L. R., Subba-Row, Y., Waller, C. W., Cosulich, D. B., Fahrenbach, M. J., Hultquist, M. E., Kuh, E., Northey, E. H., Seeger, D. R., Sickels, J. P., and Smith, J. M., Jr., *Science*, 1946, **103**, 667.

case, as judged by previous experiments and by results reported by other laboratories. Para-aminobenzoic acid is not known to be needed by chicks, but was fed as a precautionary measure. The calculated essential amino-acid content[3] of the diet satisfied the requirements of the chick for growth as described by Almquist and coworkers.[4] The chicks had an average weight of 1206 g at 10 weeks, and the group consisted of 2 males and 5 females. The chicks appeared normal in size, conformation and plumage. One of the males was observed to attempt copulation at 110 days of age. After the 155th day, 3 females and one male were kept in one group, and 2 females and one male in the other group. The males were rotated every 3rd day. Three of the females matured between the 151st and the 161st days of age, and the eggs were collected and incubated. On the 172nd day, an additional 1% of $CaCO_3$ was added to the diet to promote egg-shell formation. At 181 days of age the females averaged 2.26 kg in weight and the males 3.07 kg. The following record was obtained with the eggs:

Hen No.	1	2	3
No. of days	14	16	5
No. of eggs laid	8	9	4
No. of eggs incubated	6	4	3
Infertile	1	1	0
Dead embryo, estimated 1 to 7 days development	1	1	2
Dead embryo, estimated 17 to 21 days development	2	1	1
Live chicks hatched	2	1	0

The chicks appeared normal although they were small. They were placed on the same diet as their parents, without additional $CaCO_3$. They weighed an average of 28 g at hatching, and their average gain during the first month was 189 g.

Summary. Reproduction was obtained in chickens which were raised from hatching on a purified diet. The hatchability of the eggs was poor, but some apparently normal chicks were obtained.

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15533

Antihistamine and Antianaphylactic Effect of Hetramine, a New Synthetic Pyrimidine Compound.

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There has been increasing evidence in recent years which indicates that some of the symptoms of anaphylactic shock and other hypersensitivities are due to the liberation of histamine during an antigen antibody reaction.[1-3] For this reason much investigation has been directed toward the discovery of a substance that can counteract the effects of histamine *in vivo*.[4-8] There has been additional evidence recently that substances having antihistamine properties can do much to

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TABLE I.
Acute Toxicity of Hetramine for Mice.

Dose mg/kg	Route of administration	No. of mice	No. dead	% dead
25.0	S.Q.	10	0	0
31.25	S.Q.	18	0	0
50.0	S.Q.	10	1	10.0
62.5	S.Q.	18	4	22.2
75.0	S.Q.	10	6	60.0
100.0	S.Q.	10	10	100.0
125.0	S.Q.	18	18	100.0
200	P.O.	15	0	0
250	P.O.	23	0	0
300	P.O.	20	4	20.0
400	P.O.	20	14	70.1
500	P.O.	13	10	77.0
600	P.O.	28	22	78.6

S.Q. = subcutaneously.
P.O. = orally.

Hetramine was studied for its antihistamine and antianaphylactic properties in experimental animals. It was found to have an unusually high degree of activity in preventing histamine-induced contractions of isolated intestinal strips. Similarly, it was found to be highly effective in blocking histamine-induced shock in the guinea pig and in preventing fatal anaphylactic shock in hypersensitive animals.

The acute toxicity of hetramine for mice was determined following subcutaneous and oral administration of the compound to respective groups of animals. The LD_{50} following subcutaneous administration is between 62.5 and 75 mg/kg of mouse weight. Following oral administration, the LD_{50} was determined to be approximately 300 to 400 mg/kg. The data showing these results may be seen in Table I.

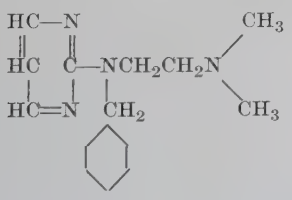
TABLE II.
Influence of Hetramine Upon Toxic Doses of Histamine.

No. guinea pigs	Hetramine pretreatment 30 min, mg/kg	Histamine mg/kg, intracardially	No. dead	% survival
6	1.0—I.P.	0.85	5	16.7
3	3.0—I.P.	0.85	3	0
6	4.0—I.P.	0.85	2	66.7
6	5.0—I.P.	0.85	1	83.3
7	6.0—I.P.	0.85	1	85.7
4	10.0—P.O.	0.85	3	25.0
5	20.0—P.O.	0.85	3	40.0
5	30.0—P.O.	0.85	3	40.0
5	40.0—P.O.	0.85	1	80.0
5	50.0—P.O.	0.85	0	100.0
8	Controls	0.85	8	0

I.P.—intraperitoneally
P.O.—per orally.

alleviate some of the symptoms associated with various types of allergies and other instances of hypersensitivity.

Hetramine which is N,N-dimethyl-N^1benzyl-N^1(a-pyrimidyl) ethylene-diamine,*



is a pyrimidine isostere of a pyridine compound which was found to have antihistamine activity.[8]

A preliminary experiment on the chronic toxicity of hetramine was carried out by testing the effect of continuous small daily doses in growing rats over a 35-day period. One group of 12 rat weanlings were put on a stock laboratory diet in which was incorporated a quantity of hetramine so that the rats consumed 50 mg/kg of rat weight per day. The concentration of hetramine in the food was adjusted daily to compensate for the growth of the rats and greater food consumption as the days passed, thus keeping the daily dosage to almost exactly 50 mg/kg per day. A second group of 12 weanlings were given a

* Synthesized by H. L. Friedman and A. T. Tolstoouhov of the chemical laboratories of the Pyridium Corporation.

TABLE III.
Efficacy of Hetramine in Preventing Fatal Experimental Asthma in Guinea Pigs Exposed to Atomized Histamine.

Hetramine mg/kg, I.P.	No. guinea pigs	No. dead	% survived
0 controls	23	22	4.3
0.156	3	3	0
0.312	3	2	33.3
0.625	7	3	57.1
1.25	4	1	75.0
1.55	6	2	66.7
2.0	6	0	100.0
2.5	4	0	100.0

I.P.—intraperitoneally.

stock diet and the drug was administered subcutaneously, twice daily, 10 mg/kg at each dose. The doses were adjusted daily to the weight of each rat. Injections were given at 9:00 a.m. and 4:30 p.m. A third group of 12 weanling rats, serving as controls, were maintained on the stock diet and given no drug.

The animals were weighed every 2nd or 3rd day and hemoglobin determinations were carried out at intervals during the 35 days of the test. These doses of hetramine were tolerated without any effect upon the growth of the young rats and without any effect on the hemoglobin, the initial average weight being 25 g in all groups and the final weight averaged 127 g in the control and 125 and 123 g in the treated groups.

The antihistamine effect of hetramine on smooth muscle strips was demonstrated in an isolated muscle bath apparatus using strips of guinea pig ileum. Kymograph tracings of the muscular contraction were made on the addition of hetramine in amounts to bring its concentration in the bath to 1 γ per cc. Histamine solution was superimposed on the hetramine to bring its concentration to 1 γ per cc. The resulting contraction if any, was recorded on the kymograph and the muscle was then washed with fresh Ringer's solution and retested with 1 γ per cc of histamine. The absence of a contraction following the addition of histamine in the presence of hetramine indicated the antihistamine effect of the latter compound. The proof of histamine activity in the concentration used was shown by the immediate contraction of the muscle on the addition of histamine after having washed out the hetramine.

As little as 1/16 of 1 γ of hetramine counteracted the muscular contractions produced by 1 γ of histamine and partial inhibition was obtained in a ratio of one part of hetramine to 32 parts of histamine.

The *in vivo* antihistamine effect of hetramine was examined by pretreating guinea pigs with various doses of the compound to be followed in 30 minutes with toxic intracardial doses of histamine. 0.85 mg/kg of histamine intracardially was found to be uniformly fatal to guinea pigs. In Table II it is shown that pretreatment with 4.0, 5.0 or 6.0 mg/kg of hetramine is effective in preventing acute toxic death of the majority of animals tested. It is also demonstrated in Table II that oral dosage is similarly effective in this respect,

TABLE IV.
Antianaphylactic Effect of Hetramine in Guinea Pigs Sensitized to Horse Serum.

No. guinea pigs shocked with 1.0 cc horse serum I.C.	Pretreatment (20 min) hetramine mg/kg I.P.	Results Intensity of reaction following shocking dose No. pigs ++++	+++	++	+	0
11	0 controls	11	0	0	0	0
6	3.5	6	0	0	0	0
8	5.0	4	1	1	1	1
8	6.0	1	1	3	1	2

I.C.—intracardially.
I.P.—intraperitoneally.
++++—fatal shock.
+++—severe shock with recovery.
++—moderate reaction with recovery
+—slight reaction with recovery.
0—no symptoms

but must be increased about 10-fold.

Exposure to atomized histamine induces a severe and frequently fatal bronchoconstriction in the guinea pig. It was found that hetramine can prevent the fatal bronchoconstriction so produced by protecting the animals with varying doses prior to exposure to a histamine atmosphere. The chamber into which atomized histamine is introduced was constructed in accordance with the methods described by Loew, *et al.*[7] It was found that vapor from 5 cc of a 0.125% solution of histamine was almost uniformly fatal to unprotected animals. This mortality could be significantly and increasingly reduced with doses of 0.3 mg/kg to 2.5 mg/kg of hetramine administered intraperitoneally. The data on this experiment are shown in Table III.

The antianaphylactic effect of hetramine was determined by sensitizing guinea pigs through intraperitoneal injection of 0.25 cc normal horse serum 14 days prior to shocking doses of 1.0 cc horse serum administered intracardially. Control animals uniformly succumbed to the shocking dose with typical symptoms of fatal anaphylactic shock. Pretreatment with intraperitoneal doses of hetramine up to 3.5 mg/kg were ineffective under our conditions. When doses of 5 mg/kg were administered 20 minutes before the shocking dose of horse serum, 50% of the guinea pigs survived and 6.0 mg/kg reduced the severity of the reaction still further, 7 out of 8 animals surviving the shocking dose. The data are shown in Table IV.

The antihistamine effect of hetramine as demonstrated by the above laboratory experiments would indicate that this drug may have a beneficial effect on some of the varied symptoms associated with hypersensitivity and physical allergies. Clinical trials are in progress to determine the effect of this compound in a variety of conditions such as, vasomotor rhinitis, hay fever, urticaria, bronchial asthma, cold allergy and other physical allergies, serum reactions and contact dermatitis.

15534

On Breeding "Wild" House Mice in the Laboratory.

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The white laboratory mouse may well be regarded as a classical example of the domestication of an animal at the hand of man. The consequences of that domestication have provided laboratory experimenters with a small docile animal the usefulness of which has been demonstrated so often as to be an established tradition. However, another consequence of that domestication has been the emergence of many genotypes distinguishable one from another by a variety of characters. This multiplicity of genotypes is a forceful reminder of the variation inherent in the species, and when, for example, a given diet produces a certain result in a certain laboratory strain of mice, we next may be expected to inquire whether (a) other genotypes will react similarly, or (b) all genotypes will do so, or (c) whether the wild species will do so. In many ways an investigation of the wild type would seem to have the greatest interest and meaning. This necessitates a laboratory supply of the wild genotype obtained either by trapping the numbers required, or by breeding them in the laboratory. For many purposes the latter method is the more preferable.

Recent investigations in the nutritional aspects of experimental epidemiology[1] have raised this question of host genotypes among

* With the technical assistance of Mr. S. A. Greenhalgh.

1 Schneider, H. A., and Webster, L. T., *J. Exp. Med.*, 1945, **81**, 359.

laboratory mice feeding on different diets. In order to extend the experiments to include the wild genotype of *Mus musculus domesticus* we attempted to establish a laboratory colony of wild house mice. The difficulties encountered, the fact that others[2] have commented on their inability to breed wild house mice in the laboratory, and the absence of published accounts on the breeding of wild house mice have prompted us to report here the measures found necessary to establish a breeding colony with mice trapped "in the wild."

Foundation Stock. The wild house mice† which formed the base of our laboratory stock were trapped in spring-door traps in the fall of 1943. Every effort was made to insure that the mice trapped were not hybridized with domestic stocks which may have escaped from laboratories or dealers. An attempt was made, too, to secure representatives from different localities in and near New York City. Thus mice were trapped in a rural community (East Northport, Long Island), in New York City apartments, in suburban Woodside, Valley Stream, and in Long Island City, Long Island. All of these mice, and all subsequent additions to a total of 35, (17 males, 18 females) were subjected to a period of quarantine of 2 weeks before being admitted to the colony. During the quarantine period each mouse was subjected to 4 successive stool cultures testing for the presence of *Salmonella enteritidis* and *Salmonella typhimurium*. All of the 35 mice examined proved to be free of these pathogenic species and in the 2½ years elapsed since the inception of the wild mouse colony no mouse typhoid disease has made its appearance. Indeed, the mice have remained healthy throughout with no signs of overt disease among them. Of the 35 mice of the original foundation stock 6 (3 males and 3 females) are still alive more than 2 years later. As far as can be judged deaths have occurred mainly through fighting, and, less often, due to what can only be described as senility. No grossly visible tumors have been observed.

Breeding trials. The initial attempts to breed wild house mice were conducted with 8 captured females and 14 captured males. With the exception of 1 female, described below, all attempts met with failure for a period of 9 months extending from October 1943 to July 1944. There would be little gain now to list the details of these failures except to state that a variety of conditions were tried involving various sizes of caging and varying periods of illumination ranging from complete darkness to a light-day of 16 hours such as Baker and Ranson[4] found necessary for the breeding of the wild field mouse *(Microtus agrestis)*. In addition various beddings of wood shavings and peat moss were tried, as well as several stock diets all of which were satisfactory in supporting the prolific breeding of laboratory mouse stocks in adjacent cages in the same room at the same time.

During this period of breeding failure a single female, pregnant on admission to the laboratory on 10/11/43, was observed to deliver a litter of 3 mice on the following day. These she consumed. Subsequently this single female, during the time of breeding failure of 7 other captured females, gave birth to 3 litters following mating with captured males. The females among these litters, however, failed to breed with captured males even when 8 weeks of age and with perforate vagina.

Anoestrous of Wild Females. The failure of captured house mice to breed was traced to the failure of the females to achieve oestrus. Captured males were potent at all times and crossing with laboratory mouse females was easily done. The reciprocal cross failed, however, although tried on numerous occasions. Further evidence of the oestrous failure of the captured wild females was obtained by daily vaginal smear examination of

[2] Andervont, H. B., and Bryan, W. R., *J. Nat. Cancer Inst.*, 1944, **5**, 143.

† The house mice of the northern United States are not truly "wild" types, but commensals (*Mus musculus domesticus*). Under favorable conditions these will adopt feral habits. The true wild type, from which *M. m. domesticus* was derived, is *M. m. wagneri*, the wild mouse of Russian Turkestan (Schwarz and Schwarz[3]).

[3] Schwarz, E., and Schwarz, H. K., *J. Mammalogy*, 1943, **24**, 59.

[4] Baker, J. R., and Ranson, R. M., *Proc. Roy. Soc. London*, B, 1932, **110**, 313; *ibid.*, B, 1932, **112**, 39; *ibid.*, B, 1933, **113**, 486.

of them for a period of 30 days. No evidence of oestrus was observed during this period. A variety of fresh green foods and a mixture of grass seeds fed as dietary supplements failed to bring the mice into oestrus.

Breeding Achieved. In May, 1944, we received a gift of 4 male and 4 female wild house mice from Dr. L. V. Heilbrunn of the University of Pennsylvania, at Philadelphia. (These mice were subsequently incorporated into our foundation stock). Dr. Heilbrunn informed us that these mice were the progeny of some wild house mice bred in his laboratory as pets. Comparison of the conditions under which these mice bred and our own failed indicated that, apart from dietary and bedding differences, the Philadelphia mice, in addition, had access to an exercise wheel. Breeding success in our own laboratory for all of our captured females was suddenly and finally achieved in July, 1944, following the introduction of exercise wheels. From that time forward we have been able to breed wild house mice at will and have prepared about 1,000 mice for experimental purposes. In this connection it is interesting to note that in their studies on the factors affecting the breeding of the field mouse *(Microtus agrestis),* Baker and Ranson[4] recorded that their cages included a "revolving wire wheel" and that "the mice made frequent use of the wire wheels." With such cages these investigators have shown clearly that the breeding of *Microtus* proceeds best in an environment which supplies a daily period of incandescent illumination of 15 hours and that shortening the light day to 9 hours almost prevents reproduction. It is the female that is chiefly affected. At the moment our own experiments, conducted with a light day of 16 hours, do not permit a decision whether for *Mus. musculus* the effect of light is direct, or indirect through its effect on activity. However, it is a fact that in our experiments a 16-hour-light day, by itself, did not result in successful breeding. Experiments are now in progress to test the effect of activity with and without light.

The Complete Breeding Technic. The technic now followed in our laboratory for the breeding of wild house mice is as follows:

Environment. A single room (18′ x 15′) is devoted to the colony. A temperature of 75°F is maintained by thermostatic control, except for summer temperatures in excess of this. Temperature rises up to 85°F are tolerated without any change in the breeding performance, but temperatures in excess of 90°F result in breeding failure. Some preliminary experiments indicated that this failure was attributable to the males, probably due to the recognized effect of excessive heat on sperm viability. The animal room is lighted from 5 a.m. to 9 p.m. (16 hours) by 2-200 watt incandescent lamps controlled by a time-clock. All daylight is excluded.

Caging. The mice are bred, 1 male to 5 females, in galvanized iron boxes 9″ x 12″ x 9″ high with galvanized mesh covers through which the water bottle tubes are inserted. Centered and 3½″ from the top edge of the box the exercise wheel-drums are suspended on steel axles extending through the sides of the box. These exercise drums are lightly constructed of copper mesh for ease of turning and are 5″ in diameter and width. Four spokes permit the mice to enter the drum easily even while it is in motion, which is often. Revolution counters attached to these exercise drums indicated that in a 24-hour period a single pair of mice, male and female, will complete 40 to 60 thousand revolutions. At this rate, by free choice the mice have run approximately 10 to 15 miles per day. When a female is observed to be pregnant she is transferred to an individual galvanized box, 7″ x 10″ x 5″, to await her litter. The young are weaned at 4 weeks of age.

Bedding. Autoclaved pine shavings are used for bedding. The shavings must be of a size large enough so that a loose mass is formed on the floor of the cage into which the mice can penetrate and hide. This is important from the standpoint of mating behavior, for if smaller sized shavings were used which readily pack down and form a firm mass so that the mice were unable to hide in it easily, then, upon mating 5 females with 1 male it was observed that the females not in heat at a given time would attack the male and often kill him upon his attempting to mate with a female which was ready to accept him. No litters were ever obtained under such conditions even if the male sur-

TABLE I.
Effects of Peat Moss and Exercise on the Breeding of Wild House Mice on a Diet of Whole Wheat Flour, Dried Whole Milk, and Salt.
(16-hr light-day.)

Diet No.	Diet description	Exercise drums	Within 60 days after mating: No. of females bred	No. of litters	Pregnancies
					%
100	Whole wheat, dried whole milk, NaCl	0	16	2	13
100	" " " " " "	+	13	11	85
259	Diet 100 plus 10% peat moss	0	15	6	40
259	" " " " " "	+	13	13	100

vived. This behavior problem could be solved either by mating the mice in single pairs, in which case the physical nature of the bedding was unimportant, or, preferably, by providing the shavings bedding in a state which allowed the mating pair to hide. Peat moss will serve as well as shavings.

Diet. At the time of the first breeding success in July 1944, following the introduction of exercise drums, all of the captured mice on hand were on a stock diet of ⅔ whole wheat, ⅓ dried whole milk and 1% NaCl (Diet 100). The bedding was peat moss. It was found that equally good results could be obtained by using pine shavings as bedding and adding 10% ground peat moss to the diet (Diet 259). This satisfactory, if unorthodox, diet served well in bringing up the numbers of the breeding stock at the same time that progeny were taken in large numbers for other experiments. At the present we are able to breed these wild mice very well on Diet 100 without the addition of peat moss. A recent experiment to test the effects of peat moss in the diet, as well as demonstrating the rôle of the exercise drums, is summarized in Table I. The females mated in this experiment were assembled into the experimental groups by dividing litter mates. There is thus no reason to attribute the obvious differences in breeding performance due to the presence or absence of exercise drums to genetic differences. The exercise effect is statistically significant; for Diet 100, $P<0.001$; for Diet 259, $P<0.01$. The effect of peat moss in improving the breeding performance, while suggestive, is not demonstrated with the same statistical adequacy ($P>0.1$).

It will be noted that on Diet 100, in the absence of an exercise drum, there were 2 pregnancies out of 16 mice bred. These 2 mice, in the absence of peat moss and exercise, probably came into oestrus by virtue of a genetic constitution which permitted this event in the absence of the environmental factors found necessary for the great number of wild house mice represented here. Such genetic variation would serve as the basis of selection of stocks independent of the environmental factors we have described as necessary, and by such means, to extend the argument, laboratory stocks may very well have been derived.

Breeding Plan. In order to maintain genetic heterogeneity the wild mouse colony has been bred in a manner so that closely related mice never form breeding pairs, but instead are crossed within the limits of the stock as much as possible. It is true, of course, that various kinds of automatic selection are going on which are slowly changing the statistical character of the wild genotype first assembled in the colony. Thus, those females which remain sterile under the conditions of the laboratory, in spite of the exercise drums, cannot contribute to the genetic composition of the succeeding generation. Such sterile genotypes will slowly be diminished in frequency. Using the per cent of pregnancies begun during the first 30 days following mating as an index of breeding performance, during the period of January 1945 to June 1946, such pregnancies rose from

56.8% to 70.2%. The average number of mice weaned per female rose in the same period from 3.69 to 4.08.

Handling of Wild Mice. All of the mice seen thus far in our colony have been as active and agile as the captured foundation stock. Special measures are necessary for handling them, for it is impossible to open a cage without having many escape. The simplest practical expedient is to perform all manipulations of the mice in a large box 18 to 20" deep with smooth sides. The escaping mice are thus confined to the larger box and may be recaught. With a little practice all of the ordinary manipulations of a mouse colony may be carried out quite easily.

Summary. The establishment of a breeding colony of wild house mice has been described. The inclusion of exercise drums in the breeding cages was found to be necessary.

15535 P

Effect of Oxygen on *P. lophurae* Infected Ducks.*

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It has been suggested that anoxia is an important factor in the mechanism of death in acute *P. falciparum* infection in man,[1] *P. knowlsi* infection in monkeys[2] and *P. lophurae* infection in ducks.[3] Furthermore it has been shown that ducks with malaria die sooner when placed in a decompression chamber than control birds kept at normal atmospheric pressure.[4] With the rapid destruction of red cells by the parasites the time may come during this disease when there is an insufficient number of red cells to carry a sufficient quantity of oxygen to supply the tissues of the host. The frequent intravenous injection of large quantities of duck red blood cells into birds that are moribund from malaria definitely prolongs their life.[5] In this study young ducks infected with *P. lophurae* have been placed in a chamber and given oxygen to determine its effect on the course of the infection.

In one experiment 10 ducks 15 days of age were inoculated intravenously with *P. lophurae* and put into the oxygen chamber. The oxygen within the tank was kept at approximately 50% concentration for 6 days at which time the amount of oxygen was increased to 75-85% for one day. Twenty similarly infected ducks were kept in batteries in the animal room. The time that death occurred in these 2 groups of ducks is shown in Experiment 1, Table I. A second group of 25 ducks 18 days of age were inoculated intravenously with *P. lophurae* infected blood. On the 4th day of the infection 10 of these were put into the oxygen chamber. The concentration of oxygen was kept at approximately 50% for 18 hours and then increased to 75% and ultimately to 85% during the following 24 hours. The 15 ducks in the control group were kept in the battery in the animal room. The time at which these birds died is shown in Experiment 2, Table I. In the third experiment 20 ducks 18 days of age were put into the oxygen chamber on the 4th day of

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1 Rigdon, R. H., *Am. J. Hyg.*, 1942, **36**, 269.

2 Rigdon, R. H., and Stratman, Thomas W. K., *Am. J. Trop. Med.*, 1942, **22**, 329.

3 Rigdon, R. H., *Am. J. Trop. Med.*, 1944, **24**, 371.

4 Rostorfer, H. H., and Rigdon, R. H., *J. Lab. and Clin. Med.*, 1945, **30**, 860.

5 Rigdon, R. H., and Varnadoe, Nona B., *Am. J. Trop. Med.*, 1945, **25**, 409.

TABLE I.
Effect of Oxygen on *P. lophuræ* Infected Ducks.

	No. of ducks in each experiment No. of ducks dead					
	Exp. 1		Exp. 2		Exp. 3	
Time	10 ducks oxygen	20 ducks control	10 ducks oxygen	15 ducks control	20 ducks oxygen	21 ducks control
5th Day—						
8 a.m.					0	1
12			0	2	0	3
4 p.m.			0	5	0	7
8			2	10	4	12
12			5	11	5	13
6th Day—						
4 a.m.			7	11	5	13
8	1	7	8	12	7	16
12	2	11	8†	12†	10	18
4 p.m.	3	15			10	18
8	3	15			10	18
12	5	15			10	18
7th Day—						
8 a.m.	7	15			12	18
12	8*	15*			13‡	18‡

* Experiment discontinued, 2 ducks survived given oxygen, 5 of the controls survived.
† " " 2 " " " " 3 " " " "
‡ " " 7 " " " " 3 " " " "

infection and 21 were kept in the battery. The oxygen concentration was kept at approximately 50% for 24 hours after which it was increased to 85-90% for the following 2 days at which time the experiment was discontinued. The time that death occurred in these 2 groups of ducks is shown in Experiment 3, Table I. The data given in Table I indicate that ducks infected with *P. lophurae* survive for a longer period when kept in an oxygen chamber containing 75 to 90% oxygen than similarly infected birds kept in batteries in the animal room.

The degree of parasitemia was followed in some of these infected ducks by counting the number of parasitized cells per 500 R.B.C. in the peripheral blood. Smears were stained with a combination of Wright's and Geimsa's stains. Our results indicate that the parasitemia is higher at the time of the peak of the infection in the birds kept in the presence of the high concentration of oxygen than it is in the control groups kept in the batteries. The total red cell counts were followed in a group of 6 young ducks kept in the oxygen chamber for 2 weeks with a concentration of approximately 50 to 75% of oxygen. These birds showed only a slight decrease in the total red cell count after being in the chamber for 5 days. The number of cells then gradually increased during a period of 48 hours and remained at a level slightly below normal for 6 days. After 2 weeks these birds were removed from the tanks and put into the regular batteries. There was no significant change in the red cell count during the following 6 days. Seven normal ducks 16 days of age were put into the chamber with an oxygen concentration of approximately 50%. After 2 days the blood from one of these birds was removed by cardiac puncture. The color index, volume index and relative cellular hemoglobin were determined. Blood was obtained from other ducks in the tank over a period of one week. Blood for the controls was obtained from similar aged birds kept in the battery. There occurred a slight increase in the color index and a slight decrease in the relative cellular hemoglobin of the ducks kept in the oxygen chambers over that of the controls.

The ducks were kept in the oxygen chamber continuously during these experiments except for the short intervals necessary to obtain

blood for the different examinations. A few of the malarial infected birds were kept in the chamber and no smears were made. The fact that these ducks survived for a longer time when given oxygen would lend support to the opinion that anemic anoxia is a significant factor in the mechanism of death in the acute forms of malaria. The results of this study would indicate that the acute forms of this disease should be treated with a plasmodicial drug and also blood transfusions and oxygen should be given to combat the anoxemia.

Summary. It has been shown that ducks injected with *P. lophurae* survive for a longer time when placed in a chamber with 75 to 90% oxygen than the controls kept in batteries in the animal room. These observations support the opinion that anoxia is a significant factor in the mechanism of death in the acute forms of malaria.

15536

Inability of Penicillin to Neutralize Dick and Schick Toxins.

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The effect of penicillin upon the toxins of various bacteria is not as yet definitely established. Neter[1] showed that penicillin did not modify the action of tetanus toxin on mice. On the other hand, Boor and Miller[2,3] demonstrated that endotoxins obtained from meningococci and gonococci caused the death of fewer mice when the mice also received injections of penicillin. Meads and his associates,[4] as well as we,[5] have observed shortening of the course of scarlet fever in most of the patients who were treated with penicillin. The fever and toxicity diminished as soon as adequate doses of penicillin were given. As a result of these clinical observations we became interested in determining whether penicillin would neutralize the erythrogenic toxin of the hemolytic streptococcus. The experiments were later extended to include the toxin of the diphtheria bacillus. The results of these investigations are reported in the present paper.

Methods and Materials. One-tenth cubic centimeter of sodium penicillin in concentrations ranging from 10 to 1000 units per cc of isotonic salt solution were mixed with 0.9 cc of standard Dick or Schick toxins. In some instances the mixtures were injected immediately and in others they were first incubated for 4 or for 24 hours. The 2 controls used with each test were: (a) the same concentration of penicillin as that in the test dose, and (b) the standard toxins, diluted in salt solution to 90% of their usual strengths. Whenever the test mixtures were incubated prior to injection, the control materials were incubated for the same period of time. The skin reactions to Dick toxin were read 24 hours after intracutaneous injection and the Schick tests after 48 hours. The degree of erythema, edema and induration was used as criteria for judging the results.

Results. Dick Tests. Altogether 67 tests with mixtures of penicillin and Dick toxin were done on 63 subjects. The results are shown in Table I. The control tests containing penicillin in salt solution gave nega-

1 Neter, Erwin, *J. Infect. Dis.*, 1945, **76**, 20.

2 Boor, A. K., and Miller, C. P., *Science*, 1945, **102**, 427.

3 Miller, C. P., and Boor, A. K., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 18.

4 Meads, M., Flipse, M. E., Barnes, M. W., and Finland, M., *J. A. M. A.*, 1945, **129**, 785.

5 Hirsh, H. L., Dowling, H. F., and Sweet, L. K., *Ann. Int. Med.*, 1946, **25**, 78.

TABLE I.

Effect of Penicillin upon Erythrogenic Toxin of *Streptococcus hemolyticus* as Determined by Dick Tests.

Conc. of penicillin in the test mixtures (units per cc)	Period of incubation (hr)	No. of tests	Results of control injection (0.9 cc Dick toxin and 0.1 cc salt solution)		Results of test injection (0.9 cc Dick toxin and 0.1 cc penicillin solution)	
			Negative	Positive	Negative	Positive
10	0	6	2	4	3	3
100	0	21	11	10	14	7
1000	0	18	3	15	3	15
100	4	3	2	1	2	1
1000	24	19	10	9	10	9
Total		67	28	39	32	35

TABLE II.

Effect of Penicillin upon Toxin of *C. diphtheriæ* as Determined by Schick Tests.

Conc. of penicillin in the test mixtures (units per cc)	Period of incubation (hr)	No. of tests	Results of control injection (0.9 cc Schick toxin and 0.1 cc salt solution)		Results of test injection (0.9 cc Schick toxin and 0.1 cc penicillin solution)	
			Negative	Positive	Negative	Positive
100	0	2	0	2	0	2
1000	0	2	0	2	0	2
10000	0	2	0	2	0	2
100	24	2	0	2	0	2
1000	24	2	0	2	0	2
10000	24	2	0	2	0	2
Total		12	0	12	0	12

tive results. Negative skin tests were observed with the Dick toxin control in 28 instances, while positive reactions were found in 39. When the combination of penicillin and Dick toxin was used, 32 were negative, and 35 were positive. The slight differences in the reactions observed are not considered significant. Incubation of the mixtures of penicillin and erythrogenic toxin did not modify the results.

Schick Tests. Twelve tests were performed on 6 subjects. The control tests with penicillin in salt solution were negative. As shown in Table II, all of the tests were positive regardless of whether the Schick toxin was mixed with isotonic salt solution or with penicillin, and regardless of whether the Schick toxin-penicillin mixtures were incubated or not.

Other Experiments with the Erythrogenic Toxin. Other methods were used to determine whether penicillin had any effect upon Dick toxin. In an experiment designed to ascertain whether penicillin would act similarly to antitoxin in the Schultz-Carlton reaction, 0.1 cc of a solution of penicillin containing 1000 units per cc was injected intracutaneously into an erythematous area of 3 patients with a florid scarlet fever rash. Blanching in this area did not occur before blanching of the general rash.

Ten patients receiving penicillin for an unrelated disease were found to be Dick positive. After they had received penicillin for one week and while penicillin was still being administered, a Dick test was again given. The test remained positive in each instance.

Summary and Conclusions. 1. When mixtures of penicillin and Dick toxin were injected intracutaneously into normal subjects along with parallel mixtures of Dick toxin and isotonic salt solution, no significant difference was observed in the number of positive Dick tests in the 2 series. Incubation of the mixtures, previous to testing, did not influence the outcome.

2. Similar results were obtained with Schick toxin.

3. Penicillin injected into an area of rash of patients with scarlet fever did not cause blanching.

4. Dick positive patients receiving penicillin for an unrelated disease did not become Dick negative after a week of penicillin therapy.

5. It is concluded that penicillin has no neutralizing effect upon Dick or Schick toxin.

We wish to thank Dr. John H. Hanks for his interest and advice.

15537

Hypertonic Sodium Chloride Solution as Serum Diluent in Agglutination Tests with *Rickettsia burneti.**

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In the course of agglutination tests employing as antigen a killed suspension of the Balkan Grippe strain of *Rickettsia burneti*,[1] it was found that a prozone effect occurred with considerable frequency. In addition, the fine granular quality of the aggregates some-

TABLE I.
Effect of Varying Concentrations of Sodium Chloride, as a Diluent for Sera, on Agglutination of a Suspension of *Rickettsia burneti.*

Convalescent serum	Conc. of diluent % NaCl	Dilution of serum 1:4	1:8	1:16	1:32	1:64	1:128	1:256	Diluent control
1	0.85	4	4	4	2	1	0	0	0
	2.5	4	4	4	4	2	±	0	0
	5.0	4	4	4	4	2	1	0	0
	7.5	4	4	4	2	1	0	0	0
	10.0	4	4	3	2	0	0	0	0
	12.5	4	4	3	2	0	0	0	0
	15.0	4	4	3	1	0	0	0	0
2	0.85	4	4	4	2	2	±	0	0
	2.5	4	4	4	4	2	1	0	0
	5.0	4	4	4	4	2	1	0	0
	7.5	4	4	4	2	1	0	0	0
	10.0	4	4	3	2	0	0	0	0
	12.5	4	4	3	2	0	0	0	0
	15.0	4	4	2	1	0	0	0	0
3	0 85	4	4	4	4	3	±	0	0
	2.5	4	4	4	4	4	2	0	0
	5.0	4	4	4	4	3	2	0	0

0—No agglutination.
±, 1, 2, 3—Increasing degrees of agglutination.
4—Complete agglutination.

* This investigation was supported through the Commission on Acute Respiratory Diseases, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, U. S. Army, and by grants from the Commonwealth Fund, the W. K. Kellogg Foundation, the John and Mary R. Markle Foundation, and the International Health Division of the Rockefeller Foundation to the Board for the Investigation and Control of Influenza and Other Epidemic Diseases for the Commission on Acute Respiratory Diseases.

1 Commission on Acute Respiratory Diseases, *Am. J. Hyg.*, 1946, **44**, 110-122.

TABLE II. Comparison of 0.85 and 5.0% NaCl Solutions, as Serum Diluents, on Agglutination of 2 Dilutions of Suspension of *Rickettsia burneti*.

Convalescent serum	Antigen dilution	Serum dilutions in 0.85% NaCl								Serum dilutions in 5.0% NaCl							
		1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
1	1/2	2	3	4	4	4	2	±	0	0	4	4	4	4	3	2	1	0	0
	1/4	0	1	4	4	2	1	0	0	0	4	4	4	3	2	1	±	0	0
2	1/2	3	4	4	4	4	4	3	1	0	4	4	4	4	3	2	1	1	0
	1/4	2	4	4	4	4	4	2	1	0	4	4	4	4	3	2	1	±	0
3	1/2	2	3	4	4	4	3	1	0	0	4	4	4	4	4	4	2	0	0
	1/4	0	1	4	4	3	2	1	0	0	4	4	4	3	2	1	0	0	0
4	1/2	4	4	4	3	1	0	0	0	0	4	4	4	3	2	0	0	0	0
	1/4	1	2	3	3	±	0	0	0	0	4	3	2	1	1	0	0	0	0
5	1/2	3	4	4	4	3	2	1	0	0	4	4	4	3	2	1	0	0	0
	1/4	1	2	4	4	3	1	±	0	0	4	4	3	2	1	1	0	0	0
6	1/2	3	4	4	4	3	1	0	0	0	4	4	4	4	3	1	0	0	0
	1/4	2	4	4	4	4	1	0	0	0	4	4	3	2	1	1	0	0	0

0—No agglutination. ±, 1, 2, 3—Increasing degrees of agglutination. 4—Complete agglutination.

times caused uncertainty in determining end points. These difficulties were most often encountered with sera of high titer and with light antigen suspensions.

As is well known, the electrolyte concentration influences the rate of antigen-antibody reactions and the ratio in which antibody combines with antigen in flocculation and agglutination tests.[2] Accordingly, the effect of varying salt concentrations on the agglutination test with Balkan Grippe antigen was investigated.

The suspension of rickettsiae used as antigen was prepared in a density comparable to Barium Sulphate Standard No. 2.[3] The sera were obtained from patients in a laboratory outbreak of Q fever caused by the Balkan Grippe strain of *R. burneti*.[4] The effect of varying salt concentrations was determined by the use of a single antigen and 3 sera serially diluted in solutions of 0.85, 2.5, 5.0, 7.5, 10.0, 12.5, and 15.0% sodium chloride, respectively.

The technic of the agglutination test[1] was as follows: sera were centrifugalized to remove particulate material and diluted serially in multiples of 2 in the respective saline solutions. To 0.2 ml of each serum dilution was added 0.2 ml of antigen dilution in 0.85% solution of sodium chloride. The tubes were incubated at 48°C for 10 minutes in a water bath, shaken for 3 minutes, returned to the 48°C water bath for 3 hours, and then placed at 4°C for 18 hours. The tubes were examined at the end of 1, 2, 3 and 21 hours. Readings were made with the aid of a concave mirror against a dark background.

The initial experiments indicated that agglutination occurred to a slightly higher titer when the sera were diluted with 2.5 and 5.0% solutions of sodium chloride (Table I). In addition, the rate of combination was somewhat more rapid with the 5.0% solution, than with the 2.5% solution. The 5.0% solution of sodium chloride was therefore selected

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[2] Boyd, W. C., *Fundamentals of Immunology*, 1943, Interscience Publishers, Inc., New York.

[3] Wadsworth, A. B., *Standard Methods*, 1939, Williams and Wilkins Co., Baltimore.

[4] Commission on Acute Respiratory Diseases, *Am. J. Hyg.*, 1946, **44**, 123-157.

as the one giving a final electrolyte concentration which was most nearly optimal. Additional experiments confirmed this finding. They indicate further that the prozone effect was reduced or eliminated when the 5.0% diluent was employed, and that a more dilute antigen could be used (Table II).

The application of these observations has made possible the use of a screen test consisting of one or 2 low-serum dilutions, for the rapid detection of rickettsial antibodies in large numbers of sera at a considerable saving of antigen.

Summary. The use of a 5.0% solution of sodium chloride as a serum diluent in agglutination tests with *Rickettsia burneti* reduced or eliminated the prozone effect and permitted the employment of a more dilute antigen.

15538

Induced *in vitro* Resistance of Staphylococci to Streptomycin and Penicillin.

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It has been recognized that certain strains of staphylococci acquire resistance to penicillin when grown in a medium containing this antibiotic.[1–6] Streptomycin[7] resistant strains can also be produced *in vitro* by exposure to this agent.[8–10]

The following study was undertaken to determine (1) the rate and degree to which various staphylococcal strains acquire resistance to streptomycin and to crystalline penicillin (G); (2) whether strains made resistant to penicillin simultaneously acquire resistance to streptomycin; (3) whether the resistant strains revert to their original sensitivity after repeated transfer in nontreated broth or when stored on dry ice for long periods of time; (4) if differences occur in the metabolic activity of the resistant and sensitive strains with respect to carbohydrate fermentation, and (5) to observe if changes in morphological characteristics occur after the organisms develop resistance to streptomycin.

Materials and Methods. The potency of the penicillin used was constant since crystalline penicillin (G) was used, however the streptomycin varied in potency from 250 to 600 units per mg. Bacto Brain Heart Infusion Broth was used as the liquid broth and standard F.D.A. agar served as the solid medium. Possible changes in the carbohydrate reactions of the cultures rendered resistant to streptomycin were investigated in Bacto Peptone Colloid Medium to which was added 0.002% phenol red and 1% of the carbohydrate. The media were inoculated from a 24-hour culture, incubated at 37°C and read after 24, 30, 48 and 120 hours.

Six strains of staphylococci obtained from different sources were used and designated as

1 Abraham, E. P., Chain, E., Fletcher, C. M., Gardner, A. D., Heatley, N. G., Jennings, M. A., and Florey, H. W., *Lancet*, 1941, **2**, 177.

2 Rommelkamp, C. H., and Maxton, T., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 386.

3 McKee, C. M., and Houek, C. L., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 33.

4 Demerec, M., *Proc. Nat. Acad. Sci.*, 1945, **31**, 16.

5 Todd, E. W., Turner, G. S., and Drew, L. G., *Brit. Med. J.*, No. 4386, 1945.

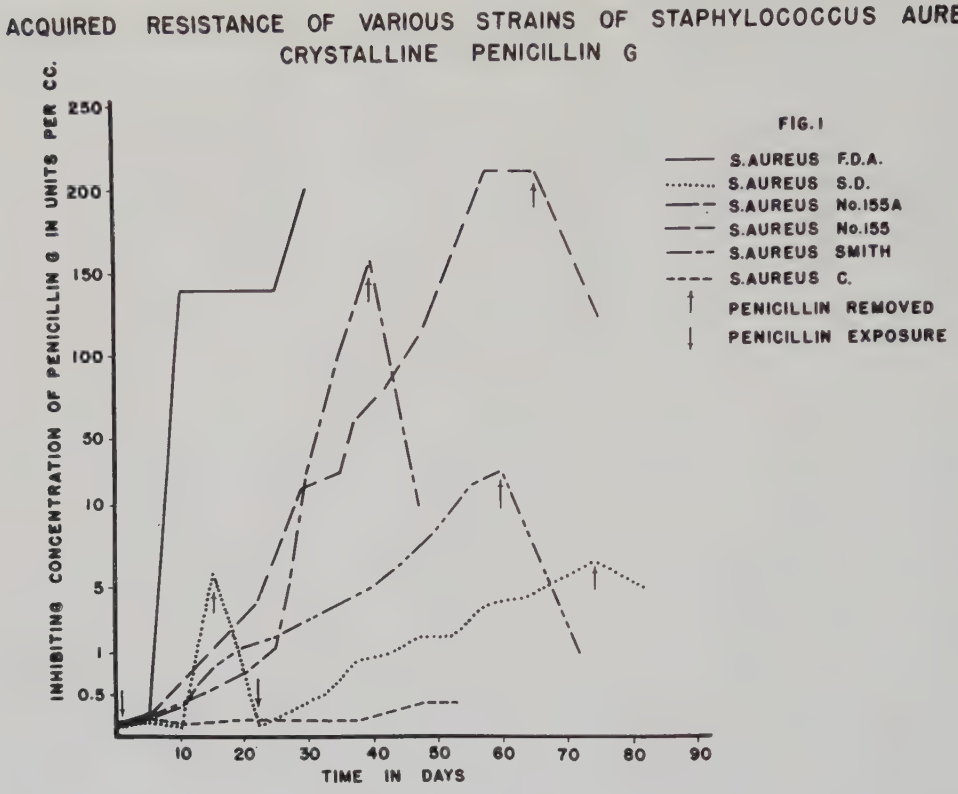
6 Rake, G., McKee, C. M., Hamre, D. M., and Houck, C. L., *J. Immunol.*, 1944, **48**, 271.

7 Schatz, A., Bugie, E., and Waksman, S. A., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 66.

8 Waksman, S. A., Reilly, H. G., and Schatz, A., *Proc. Nat. Acad. Sci.*, 1945, **31**, 157.

9 Youmans, G. P., Williston, E. H., Feldman, W. H., and Hinshaw, H. C., *Proc. Staff Meet., Mayo Clinic*, 1946, **21**, 126.

10 Miller, C. P., and Bohnhoff, M., *J. A. M. A.*, 1946, **130**, 485.



S. aureus Smith, S. aureus F.D.A., S. aureus No. 155, S. aureus No. 155A, S. aureus S.D., and *S. aureus C.*

Regression of induced resistance to the antibiotics was studied by freezing the fast strains and storing them on dry ice for from 9 to 12 months. Upon removal they were grown through several transfers before testing for resistance.

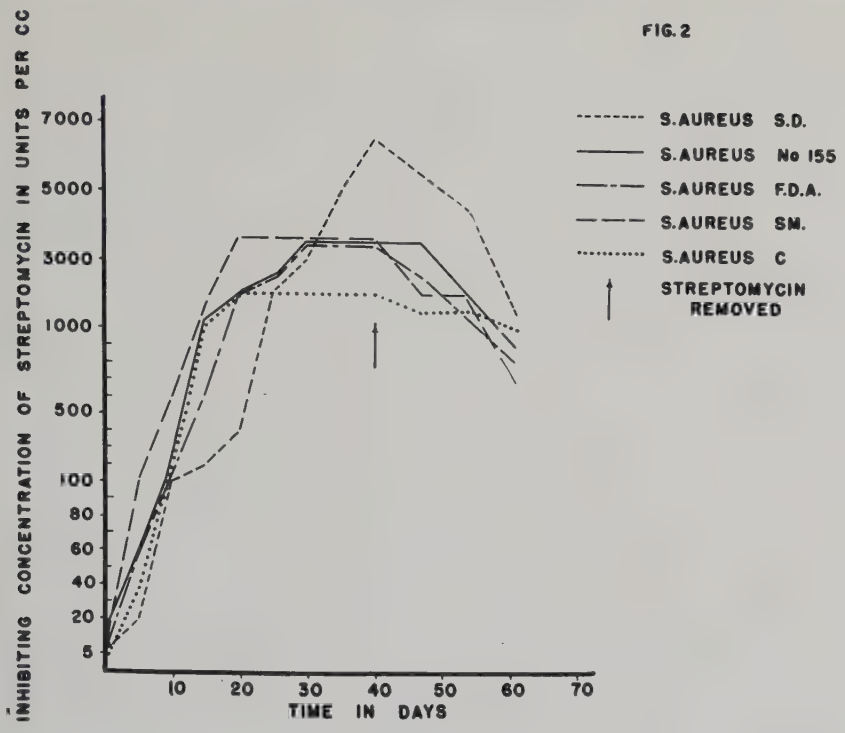
The sensitivity of each organism to the antibiotics was determined by the following method: A series of dilutions of the test agent in sterile distilled water was prepared in such a manner that they were 10 times more concentrated than the final dilution desired. To 1 cc of each dilution was added 9 cc of seeded culture medium containing a 10^{-5} dilution of a 24-hour culture of the test organism. The tubes were incubated at 37°C for 24 hours and examined for turbidity. The minimal concentration of the antibiotic in units per cc which resulted in complete inhibition of growth was taken as the inhibiting concentration.

Resistance was produced by transferring each culture into broth containing one-half of its inhibiting concentration of the antibiotic. After 5 such daily transfers, the bacteriostatic concentration for the culture was redetermined, and the drug concentration increased so as to equal one-half of the new inhibiting level. This procedure or a slight modification, was continued until no further significant change in resistance was evident. Control cultures were maintained by daily transfer in untreated broth.

Results. Induced Resistance. All of the cultures exposed to penicillin and streptomycin as described above decreased in sensitivity to the antibiotics. The degree to which resistance was acquired depended upon the strain and the duration of exposure. Thus, 3 strains of *Staphylococcus aureus* acquired a resistance to penicillin of at least 1500-fold within 10 to 52 days; whereas one culture became only 4 times more resistant when exposed in the same manner (Fig. 1). Furthermore, the rate at which resistance was ac-

ACQUIRED RESISTANCE OF VARIOUS STRAINS OF STAPHYLOCOCCUS AUREUS TO STREPTOMYCIN

FIG. 2



quired also varied as evidenced by the fact that one strain *(S. aureus F.D.A.)* developed a resistance of approximately 1000-fold in 10 days, whereas other strains required from 35 to 45 days to withstand the same increase in penicillin concentration.

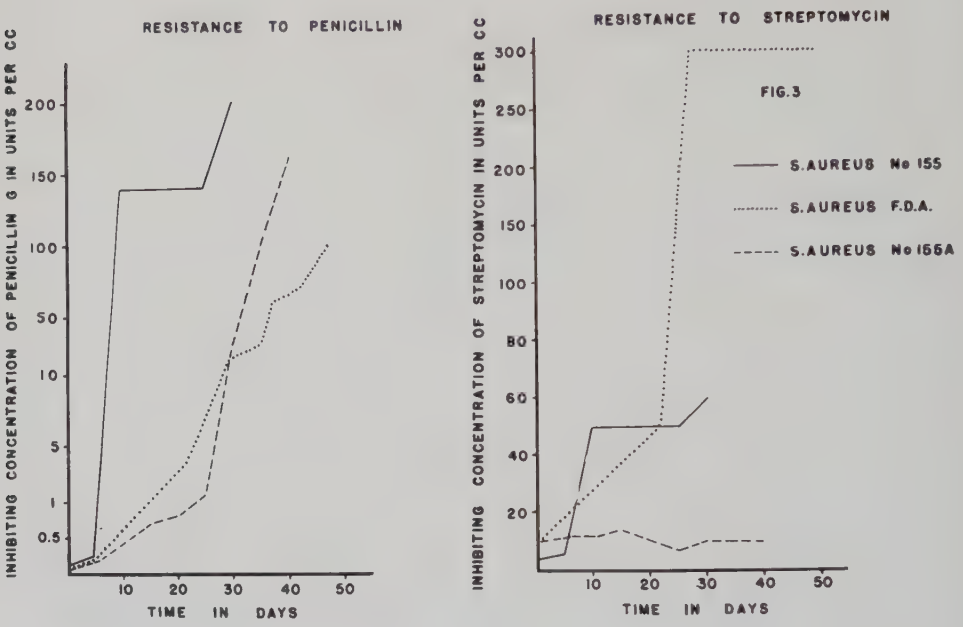
A similar resistance developed when the original cultures were exposed to increasing concentrations of streptomycin (Fig. 2), but showed less variation than when exposed to penicillin. It seemed to occur more rapidly, but to a lesser degree than to penicillin. In all cases resistance increased rapidly until the organisms tolerated approximately 300 to 700 times as much streptomycin as originally. It was of interest to note that none of the resistant strains destroyed streptomycin indicating that increased resistance is not due to the formation of a streptomycin-destroying enzyme.

Regression of Induced Resistance. In order to determine whether a resistant strain would revert to its original sensitivity when cultured in broth free of penicillin, strain *S. aureus S.D.* which had acquired a resistance of 60-fold in 15 days was transferred daily in nutrient broth over a 7-day period. The results show that with this strain resistance was lost within 7 days (Fig. 1). Upon re-exposure to penicillin the culture became resistant to the same degree, requiring however, a longer period of time. Similar tests were performed with *S. aureus No. 155A, S. aureus Smith* and *S. aureus No. 155* after these cultures acquired an increased resistance of 1600-, 300- and 2100-fold respectively.

All of the penicillin-resistant strains tested showed some decrease in resistance upon transfer in nutrient broth.

When the streptomycin-resistant strains were transferred in a similar manner they also became more sensitive to the antibiotic. Over the 21-day period during which these strains were followed in broth they showed less variation in the rate at which resistance regressed than was observed with the penicil-

ACQUIRED RESISTANCE TO PENICILLIN AND STREPTOMYCIN OF STAPHYLOCOCCAL STRAINS EXPOSED TO CRYSTALLINE PENICILLIN G



lin-resistant strains.

When stored on dry ice for from 9 to 12 months 3 of the 5 strains which had acquired resistance to penicillin were found to be more sensitive. The resistance of strains S.M., S.D. and No. 155A was reduced from 300-, 65- and 1600- to 40-, 3.5- and 70-fold respectively when stored for this period of time. On the other hand, strains F.D.A. and No. 155 remained practically unchanged. It is notable that the strains which retained their resistance were those which showed the greatest degree of increased resistance during exposure to penicillin. In contrast to the foregoing findings all streptomycin-fast strains retained their resistance to this antibiotic when stored at a low temperature for long periods of time.

Cross Resistance. During the period in which the staphylococci were being made resistant to penicillin they were also tested for sensitivity to streptomycin. A change in the inhibitory effect of penicillin on the streptomycin-fast strains was also investigated. Under these conditions it was observed that *S. aureus F.D.A.* and *S. aureus No. 155* while acquiring resistance to penicillin had simultaneously become 15 and 30 times more resistant to streptomycin. These strains had also shown the greatest increase in resistance to penicillin. Three additional strains did not show this increase in resistance. For simplicity the results observed with 3 representative strains are presented (Fig. 3). In contrast, all 5 streptomycin-resistant strains remained sensitive to penicillin.

Morphology. Cultures acquiring resistance to commercial penicillins have been reported to show morphological changes,[6,11,12] marked pleomorphism,[6,11] loss of pigmentation,[6,11] and slowing of metabolic reactions.[1,6,11,12] Similar morphological changes and pleomorphism as have been described by others were observed in the staphylococcal strains made resistant to crystalline penicillin (G).

Studies on the growth characteristics of the streptomycin-resistant strains showed no

[11] Gardner, A. D., *Nature*, 1940, **146**, 837.

[12] Spink, W. W., Ferris, V., and Vivino, J. J., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 210.

TABLE I.
Carbohydrate Reactions of Streptomycin Resistant Staphylococcal Strains.

Carbohydrate	*S. aureus S. M.* Control Time in hr 24	30	48	120	*S. aureus S. M.* Streptomycin Resistant Time in hr 24	30	48	120	*S. aureus F. D. A.* Control Time in hr 24	30	48	120	*S. aureus F. D. A.* Streptomycin Resistant Time in hr 24	30	48	120
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	±	+	+	+
Lactose	—	—	—	—	—	—	—	—	+	+	+	+	±	+	+	+
Maltose	+	+	+	+	—	—	—	+	+	+	+	+	±	+	+	+
Sucrose	+	+	+	+	—	—	±	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	—	±	+	+	+	+	+	+	+	+	+	+
	S. aureus Cameron								*S. aureus S. D.*							
Glucose	+	+	+	+	±	+	+	+	+	+	+	+	±	±	±	+
Lactose	+	+	+	+	±	+	+	+	+	+	+	+	±	+	+	+
Maltose	+	+	+	+	±	±	+	+	+	+	+	+	±	±	±	+
Sucrose	+	+	+	+	±	+	+	+	+	+	+	+	±	+	+	+
Mannitol	—	—	—	—	—	—	—	—	+	+	+	+	±	+	+	+
	S. aureus No. 155															
Glucose	±	+	+	+	±	+	+	+								
Lactose	±	±	+	+	±	±	±	+								
Maltose	+	+	+	+	+	+	+	+								
Sucrose	+	+	+	+	+	+	+	+								
Mannitol	+	+	+	+	+	+	+	+								

+ Acid. ± Some acid. — No acid.

marked differences in the appearance of the broth cultures or colony formation on agar, except for a loss of pigmentation by the resistant *S. aureus F.D.A.* and *S. aureus Smith.*

Carbohydrate Fermentation. At peak resistance the streptomycin-resistant staphylococcal cultures showed no change in the sugars fermented (Table I), but an appreciable increase in the time required for the fermentation was observed. Two weeks after the cultures had been returned to broth without streptomycin they were retested, and with the exception of *S. aureus No. 155* which continued slow in fermenting lactose, produced acid at the same rate as the control cultures.

Summary. 1. Under certain conditions strains of *S. aureus* develop resistance *in vitro* to streptomycin or to penicillin (G). 2. Greater variation occurs in the degree and rate at which resistance is acquired to penicillin than to streptomycin. 3. All of the streptomycin and penicillin-resistant strains studied showed some decrease in resistance upon transfer in broth free of the antibiotics. 4. Two of the penicillin-fast strains increased in resistance to streptomycin whereas the streptomycin-fast strains remained sensitive to penicillin. 5. Some streptomycin-fast staphylococcal strains show a change in pigment production and a reduction in the rate of carbohydrate fermentation.

15539

Effect of Concentrated Hyperimmune Rabbit Serum on Circulating Agent in Louse Borne Typhus.

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Immune serum has been used by a number of workers in the treatment of rickettsial diseases. A recent report by Yeomans, Snyder and Gilliam reviewed the previous work and reported upon the use of a concentrated hyperimmune rabbit serum in the treatment of 25 cases of epidemic typhus fever studied in Cairo, Egypt.[1] These authors concluded that hyperimmune antityphus rabbit serum had a favorable therapeutic effect on the course of typhus if treatment was started in the first 3 days of the disease. Although serum treatment seemed to have reduced the mortality in the group of patients who received serum on the 4th, 5th, or 6th day of illness, the value of serum therapy for late cases could not be determined.

Of the 25 cases studied by Yeomans, Snyder and Gilliam, strains of epidemic typhus were isolated in guinea pigs by the authors in 20 instances. In 11 of these cases, attempt at strain isolation was made immediately before and again at variable times after the administration of the serum. Since the method employed for strain isolation was identical in each instance, it was possible to ascertain the effect of the serum upon the circulating agent. Furthermore, the nature of this effect could be determined, within certain limits, by comparing the period of incubation in the inoculated guinea pigs in the pre- and postserum isolations.

Method. Thirty cc of blood were withdrawn from the patient. The blood was kept in ice and usually was inoculated into guinea pigs within 2 hours after withdrawal. After coagulation, the serum was removed and the clot was ground up in a mortar with alundum. This material was then suspended in 10 cc of physiological saline. After the alundum sedimented, 5 cc of the supernatant fluid were inoculated intraperitoneally into each of 2 male guinea pigs. The temperatures of the guinea pigs were taken for several days prior to inoculation in order to obtain the normal level and for 21 days after. A temperature of 104°F or higher was considered as fever.

Table I records the pertinent data in this study. These data indicate that in 11 cases of epidemic typhus fever treated with hyperimmune rabbit serum, it was possible to isolate the agent in guinea pigs immediately before and again at variable periods after the administration of serum. This was true when the postserum isolations were made immediately after and up to 114 hours after the administration of serum. Even though circulating agent was still present after the administration of serum, some deleterious effect of the serum upon the agent was evident as judged by the prolongation of the period of incubation in the postserum isolations. There were 2 exceptions. In Case 24, blood was drawn for postserum isolation 3 minutes after the administration of serum. The incubation period in the inoculated guinea pigs was reduced 2 days. In Case 20, in an isolation made 21 hours after the administration of serum the period of incubation was prolonged by 3 days, while with the isolation made after 114 hours, this period was reduced 2 days. It should be noted that in 6 cases one guinea pig each in the postserum isolations failed to react when observed over a 21-day period. This failure to react may have been due to an inapparent infection which is characterized by no evident symptoms of disease but is followed by the appearance of specific antibodies, or by a missed infection characterized by no symptoms, or the appearance of specific antibodies.[2] These possibilities may occur normally after the inoculation of

* Member, United States of America Typhus Commission.

1 Yeomans, A., Snyder, J. C., and Gilliam, A. G., *J. A. M. A.*, 1945, **129**, 19.

2 Plotz, H., Wertman, K., and Bennett, B. L., Proc. Soc. Exp. Biol. and Med., 1946, **41**, 76.

TABLE I.

Case No.	Day of disease when treatment began	Avg incubation preserum isolation, days	Amt of serum administered between isolations	Interval between isolations	Avg incubation postserum isolation, days	Difference between pre- and post-serum incubation	Total amt serum administered (cc)
6	3	11½	39 iv—20 im	14 hr	14	Prolonged 2½ days	239
11	4	11½	58 iv	15 "	14*	" 2½ "	58
13	4	11½	51 iv	19 "	20*	" 8½ "	51
14	4	10	20 iv—37.5 im	16½ hr	12	" 2 "	218
15	4	13	126 iv	5 mm	17*	" 4 "	512
16	4	10	85 iv	11 hr	12*	" 2 "	491
19	5	7	40 iv—40 im	Immediately after	11*	" 4 "	120
20	5	12	78 iv	1st isol, 21 hr 2nd isol, 114 hr	15 10	" 3 " Reduced 2 "	78
22	5	10½	30 iv—60 im	7 hr	16*	Prolonged 5½ "	260
24	6	11½	59 iv	3 min	9½	Reduced 2 "	59
25	6	12	40 iv—38 im	7 hr	14½	Prolonged 2½ "	238

* One guinea pig of each of these groups did not react when observed 21 days.
The case numbers employed here correspond to those used by Yeomans, Snyder and Gilliam.[1]
Strain isolation for Case 6 and 13 is incorrectly recorded by Yeomans, Snyder and Gilliam.[1]

infectious material into guinea pigs. Finally, there may be a rickettsiostatic effect of the serum upon the circulating agent. The precise reason is not known for antibody studies were not made on these 5 specimens of guinea pig convalescent serum.

Seven of the cases (6, 14, 15, 16, 19, 22 and 25) received further doses of serum after the second isolation of the agent and hence, it is impossible to determine the possible effect of the early administration of serum upon the clinical course of the disease. However, Cases 11, 13, 20 and 24 were of particular interest since the total amount of serum administered occurred between the 2 strain isolations.

Cases 11 and 13 were recorded[1] as being of moderate severity, showing slight prostration, central nervous system involvement, cardiovascular changes or mild complications. The only effect observed after the administration of 58 cc and 51 cc of serum, respectively, was to prolong the incubation period in the guinea pigs when isolations were made 15 and 19 hours after serum treatment. Case 24, who received 59 cc of serum, was characterized as being a severe typhus with definite prostration, central nervous system involvement, cardiovascular changes or serious complications (nitrogen retention). When an isolation was attempted 3 minutes after the administration of serum, the period of incubation was reduced from what was found for the preserum isolation. In Case 20, which was characterized as being of such severe illness that a fatal outcome was expected at some point in the clinical course, the administration of 78 cc of serum had a slight effect upon the agent when isolation was made after 21 hours and none after 114 hours.

Since a favorable effect of the serum on the course of the illness was obvious from the clinical observations, the question arises as to what this effect may be attributed. It is evident that the serum did not have a rickettsiocidal effect upon the agent, for in 11 instances we were able to isolate the agent after the administration of serum. The serum may have had a rickettsiostatic effect. In favor of this hypothesis would be the prolonged incubation period in the guinea pigs in the postserum isolations. The final and

most likely hypothesis is that the serum exerted beneficial clinical effect because of an antitoxic effect. It has been shown that a "toxic factor" is associated with the epidemic and murine strains of typhus rickettsiae cultivated in yolk sac cultures and that this "toxic factor" can be neutralized specifically by convalescent serum.[3-6] This may explain why such relatively small amounts of serum as were employed in Cases 11, 13, 20 and 24 exerted a beneficial clinical effect. The hyperimmune rabbit serum used in these cases was prepared with infected yolk sac cultures.

Conclusion. 1. In 11 cases of epidemic typhus treated with hyperimmune rabbit serum, strains were isolated immediately before and again at variable periods after the administration of serum. 2. The only effect observed was that in the postserum isolations the period of incubation in the inoculated guinea pigs was usually, but not always, prolonged. 3. While the effect of the serum upon the agent may be rickettsiostatic, it is believed that a more likely explanation of the favorable clinical effect was due to its antitoxic effect.

3 Gildemeister, E., and Haagen, E., *Deut. Med. Wchschr.*, 1940, **66**, 878.

4 Bengtson, I. A., Topping, N. H., and Henderson, R. G., Nat. Inst. of Health, Bull. No. 183, 1945, p. 25.

5 Henderson, R. G., and Topping, N. H., Nat. Inst. of Health, Bull. No. 183, 1945, p. 41.

6 Hamilton, H. L., *Am. J. Trop. Med.*, 1945, **25**, 391.

15540

Propagation of Hog Cholera Virus in Rabbits.

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The literature in regard to negative results in attempts to transmit hog cholera virus to hosts other than swine, is too voluminous to be cited. The only evidence of actual transmission of the virus in other hosts has been furnished by Zichis[1] who was able to propagate the virus for 10 passages in domestic sheep. Cultivation of the virus in hog tissue outside the body of the animal has been reported by Hecke[2] and TenBroeck.[3]

Even though all evidence indicated that hog cholera would be a difficult virus to adapt to other hosts, it seemed that in view of the knowledge gained with other viruses—such as: poliomyelitis,[4] dengue fever,[5] Colorado tick fever[6,7] and rinderpest,[8-11] the problem should be reinvestigated.

For some time attempts have been made in this laboratory to adapt hog cholera virus to some host other than swine. In accordance with the experience of most previous investigators most of our attempts ended in failure. Recently, however, by using an alternating passage method similar to that reported by Baker[10] in his work with rinderpest virus in rabbits, we have obtained results indicating that hog cholera virus may be successfully passed for a number of generations in rabbits.

1 Zichis, J., *J. Am. Vet. Med. Assn.*, 1939, **95**, 272.

2 Hecke, F., *Zentralbl. Bkt. Abt. I, Orig.*, 1932, **126**, 517.

3 TenBroeck, C., *J. Exp. Med.*, 1941, **74**, 427.

4 Armstrong, C., *Pub. Health Rep.*, 1939, **54**, 2302.

5 Sabin, A. B., and Schlesinger, R. W., *Science*, 1945, **101**, 640.

6 Florio, L., Stewart, M. D., and Mugrage, E. R., *J. Exp. Med.*, 1944, **80**, 165.

7 Koprowski, H., and Cox, H. R., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 320.

8 Nakamura, J., Wagatuma, S., and Fukusho, K., *J. Jap. Soc. Vet. Sci.*, 1938, **17**, 185; English Summary, pp. 25-30.

9 Kunert, H., *Dtsch. tierarztl. Wschr.*, 1938, **46**, 487.

10 Baker, J. A., *Am. J. Vet. Res.*, 1946, **7**, 179.

11 Shope, R. E., Griffiths, H. J., and Jenkins, D. L., *Am. J. Vet. Res.*, 1946, **7**, 135.

TABLE I.
Summary of Hog Cholera Virus Passages in Rabbit Spleen.

Rabbits				Swine	
Rabbit passage*	No. inoculated	No. with febrile reaction	Highest temp, °F	No. injected	Results
1	7	4	104.8		
3	2	2	104.7		
5	4	3	104.6		
6	3	3	105.2	2	1 sacrificed when sick on 6th day. 1 survived with development of immunity (see text).
8	4	3	105.1	2	2† died on 18th day with typical hog cholera lesions.
9	4	3	104.8	1	1† died on 18th day with typical hog cholera lesions.
11	4	2	105.6	2	2 survived. 1 showed delayed febrile reaction.

* Refers to number of passages counting from the 6th alternating pig-to-rabbit to pig-to-rabbit passage.

† Normal swine placed in the same pen with these animals contracted hog cholera and died from the disease.

The presentation of experimental data is the subject of this report.

Material and Methods. The hog cholera strain was the stock virus used by the Lederle Laboratories in St. Joseph, Missouri* for the production of hog cholera virus vaccine and immune serum. Pigs were bought from local farmers whose premises were known to be free from hog cholera and who employed no hog cholera vaccines. The pigs were obtained immediately after weaning and care was taken to ascertain that none of the animals were exposed to hog cholera or were sick prior to purchase. Upon arrival in this laboratory, the pigs were kept in strict quarantine for

* We are indebted to Mr. Frank Cooper, of the St. Joseph plant, for his cooperation in the initial stages of this work.

FIG. 1
TEMPERATURE CURVE OF RABBIT
INJECTED WITH THE EIGHTH RABBIT SPLEEN-PASSAGE VIRUS

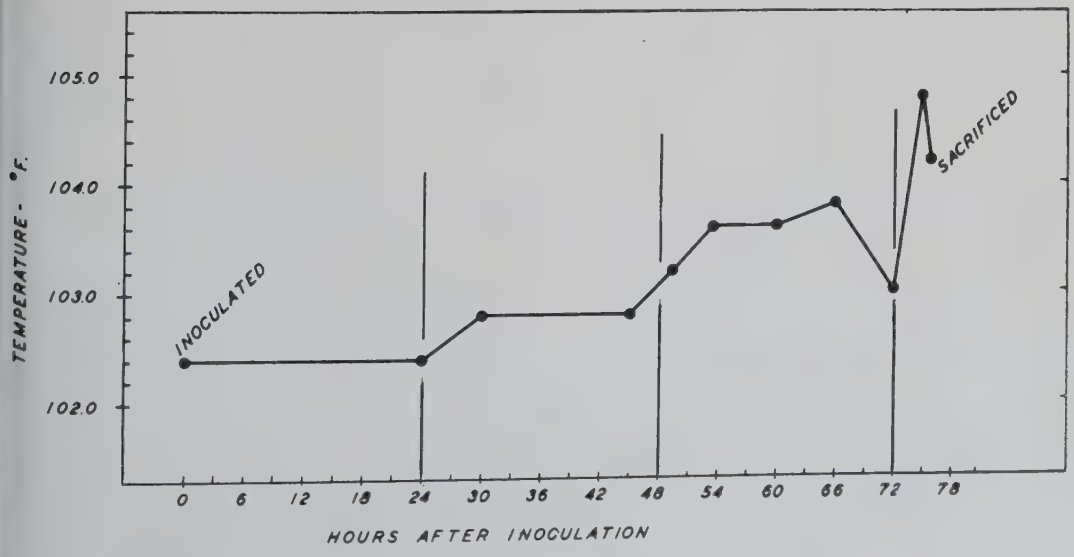
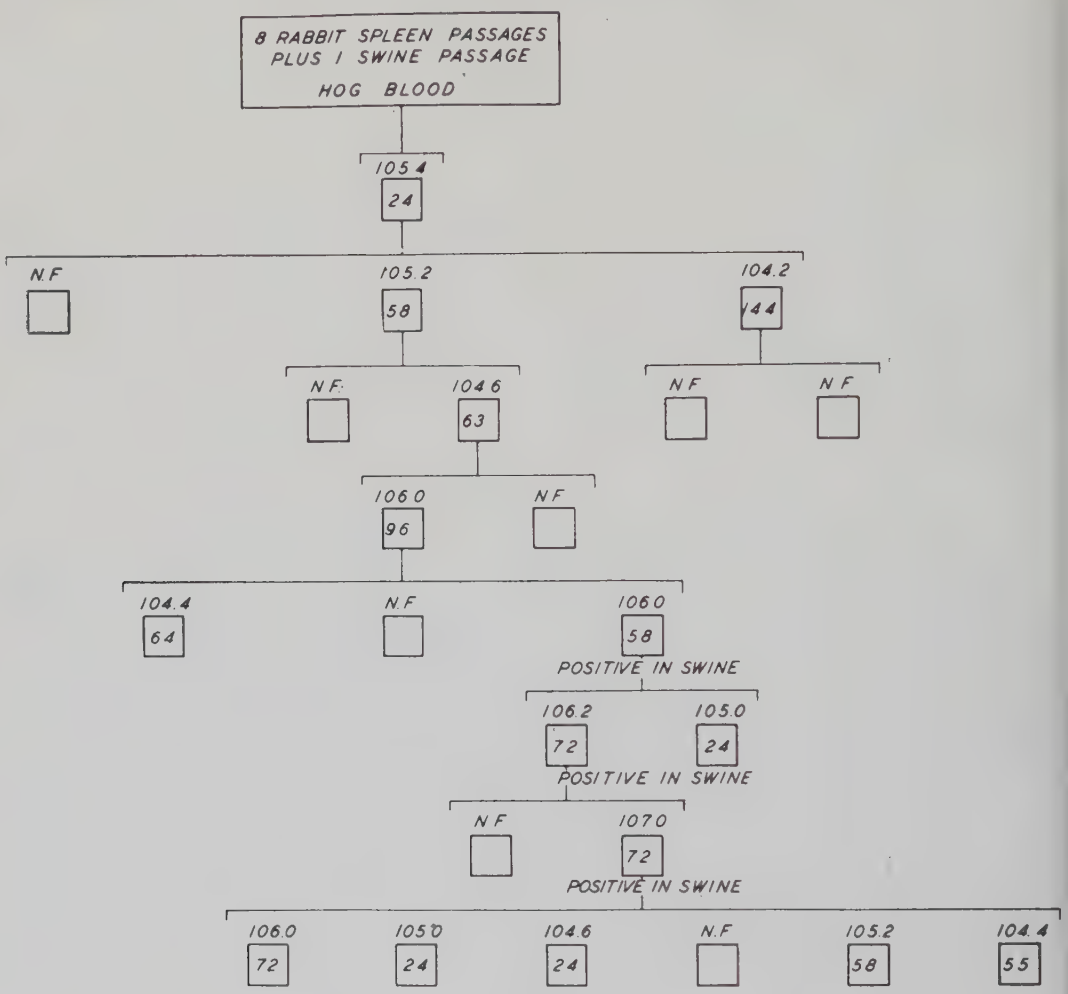


FIG. 2

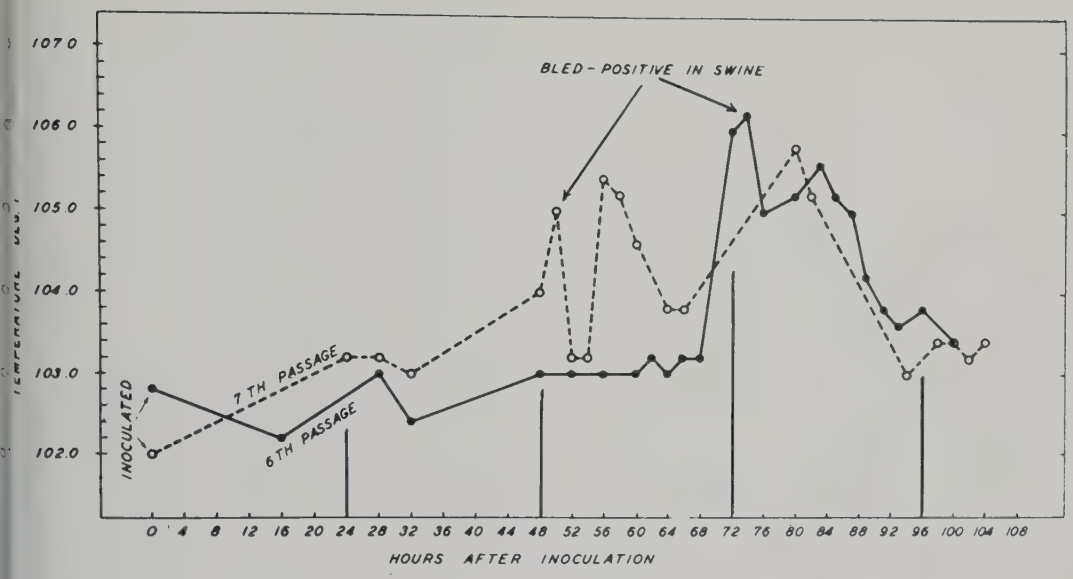
SUMMARY OF HOG CHOLERA VIRUS PASSAGES IN RABBIT BLOOD



2 weeks and released for experimental use only if signs of disease were absent in the entire lot. Inoculated pigs were housed in individual pens under strict isolation so that any possibility of cross-infection was ruled out. White rabbits of the "New Zealand" strain were used throughout the work. The animals were 5-8 lb. in weight and were kept individually in cages. The pigs were inoculated intramuscularly in the groin and the rabbits were inoculated in the marginal ear vein. Rectal temperatures were taken on the animals twice daily, at 8:30 a.m. and 4:30 p.m., unless otherwise stated.

Experimental. Infected hog spleen, derived from an animal which was sacrificed on the

FIG 3
TEMPERATURE CURVES OF RABBITS
INJECTED WITH THE SIXTH AND SEVENTH RABBIT BLOOD-PASSAGE VIRUS



6th day after inoculation with hog cholera virus, was made into a 10% suspension in saline in a TenBroeck grinder. The suspension was centrifuged for 5 minutes at 1000 r.p.m. and 2 ml of the resulting supernatant were inoculated intravenously into a rabbit. The rabbit showed no elevation of body temperature but was sacrificed on the 3rd day after inoculation and a 10% suspension of its spleen inoculated into a pig. The latter showed fever up to 105-106°F on the 2nd, 3rd and 4th days after inoculation and was sacrificed on the 5th day. A suspension of its spleen, in turn, was injected into a rabbit. The virus was transferred by means of this technic of alternation between the hog and one or 2 rabbit passages until it had been carried through 6 intermittent rabbit passages. Out of 7 rabbits injected with the 6th passage virus, 3 showed slight elevation of temperature on the 2nd day after inoculation, and were sacrificed at that time. Their spleens were pooled and a suspension of them was injected intravenously into another group of rabbits. These animals also reacted with a slight fever and were sacrificed in turn, and the virus was thus transferred for 2 more rabbit passages. At this stage, none of the rabbits showed increased temperatures on routine checking and no virus was detected in their spleens by subinoculation of swine. It thus became obvious that the virus was either merely transferred from one rabbit to another without proliferation or that the technic of taking temperatures only twice during the daytime, following the technic used by Baker[8] in his work with rinderpest virus, was inadequate. In order to test the latter possibility, a rabbit spleen suspension, representing the 3rd rabbit passage, was injected into 3 rabbits, temperatures of which were taken every 2-3 hours throughout the 24-hour period. All 3 rabbits reacted with a febrile response on the 2nd night after inoculation. Curiously enough the elevated temperature lasted only 2-3 hours after which it started to drop. The animals were sacrificed during this febrile period and a pool of their spleens when injected into swine proved to contain hog cholera virus. By adopting the above technic of taking temperatures every 2 or 3 hours, day and night, the virus was carried for 12 continuous rabbit passages by spleen transfer. Febrile response was the only sign of infection in the rabbits. The data are summarized in Table I. A

typical temperature curve of a rabbit injected with the 8th rabbit passage spleen-virus is shown in Fig. 1.

Proof for Identity of the Virus. A pig injected with the 6th rabbit passage virus reacted with a 6-day febrile period but survived, and was subsequently placed in contact with 2 pigs which sickened, following injection with the original hog strain of Lederle blood virus. These animals were kept in an uncleaned pen with 2 normal contact control pigs. The 2 animals injected with the hog strain of the virus died and the 2 control swine showed fever after 5 and 6 days of contact respectively, and subsequently died on the 12th and 19th days. On autopsy, typical lesions of hog cholera were observed in the dead swine whereas the pig, injected with the 6th rabbit passage virus, remained asymptomatic during the entire 30-day observation period. On the 34th day this pig was injected with 2 ml of a 1:100 dilution of swine blood infected with hog cholera virus (Lederle stock strain). Normal swine injected simultaneously came down with typical symptoms of hog cholera whereas the pig which was previously injected with the 6th rabbit passage virus remained afebrile and symptom-free during the whole observation period.

Swine injected with the 8th and 9th rabbit passage spleen-virus (Table I), were autopsied after death and typical lesions of hog cholera were observed.

Propagation of the Virus in Rabbits by Injections of Infectious Blood. A pig injected with a spleen suspension of the 8th rabbit passage virus was bled when febrile on the 5th day after inoculation and 2 ml of its defibrinated blood were injected into the ear vein of a rabbit. The rabbit became febrile (105.4°F) 20 hours after inoculation at which time 20 ml of blood were taken by heart puncture. The blood was defibrinated and 2 ml were injected intravenously into each of 3 rabbits. Two of the animals became febrile and their blood was passed to another group of rabbits. By means of this technic, the virus has been propagated for 8 passages in rabbits. The data are summarized in Fig. 2. It may be observed that not all rabbits inoculated with the infected blood reacted with fever. However, in the later passages more animals showed a febrile reaction, and the temperature peaks reached higher levels and persisted for longer periods than in those rabbits inoculated with the spleen-passage virus, although in the latter case it was difficult to ascertain how long the febrile period lasted because the animals were sacrificed immediately after the supposed peaks of fever were reached.

In Fig. 3 are shown temperature curves of rabbits inoculated respectively with the 6th and 7th rabbit blood-passage virus. It may be observed that the temperature of the rabbit inoculated with the 6th passage virus started to rise between the 68th and 70th hour after inoculation, reached its peak on the 74th hour when the animal was bled and remained above normal for 16 hours. Blood of this rabbit produced a typical clinical picture of hog cholera in an inoculated pig which died on the 13th day after inoculation. On the other hand, the temperature curve of the rabbit injected with the 7th rabbit blood passage virus followed a slightly different course. A temperature of 105.0° was reached on the 50th hour after inoculation when the animal was bled. Then the temperature curve dropped rather suddenly and remained at a 103.2° level during 2 hours, returning back to a 105.4° level at the 56th hour after inoculation. During the next 26 hours the temperature was either at a subfebrile (103.8°) level or at a febrile level (105.8°), returning back to normal at the 94th hour after inoculation and remaining normal until the end of the observation period. Blood of this rabbit injected into a pig again produced a clinical picture of hog cholera. Up to now, no symptoms other than a febrile response were observed in rabbits injected with the rabbit blood-passage virus.

Discussion. The data presented above indicate that hog cholera virus has been successfully passed through several generations in rabbits by the use of an alternating passage method. However, it was apparently also essential to take temperatures of the rabbits frequently day and night during the entire observation period—and to make pas-

age transfers promptly at the time the febrile esponse occurred.

It may be of interest to mention here that ther investigators[10,12–16] have used the con-ept of alternating passages to maintain, modi-y, reactivate or adapt viral or rickettsial gents to other hosts or tissues.

It is conceivable that it may not be essen-ial to use the alternating passage method o pass hog cholera virus in rabbits since in ork with rinderpest virus, Nakamura and olleagues[8] were able to adapt the virus to abbits and maintain it for 166 consecutive erial passages without going back to the riginal host. Edwards[17] also reported success in maintaining rinderpest virus in rabbits for a period of at least 14 months by going directly from the calf to the rabbit and by making successive intravenous transfers in rabbits every 2-7 days. On the other hand Baker[1] found it necessary to use the alternating passage method to achieve the same object. The difference in results obtained by various investigators could be due to the fact that different breeds of test animals or different strains of virus may have been employed.

Summary. Hog cholera virus has been carried for 12 consecutive passages in rabbits by using infected rabbit spleen as transfer material. Starting from the 8th rabbit spleen passage, the virus was passed back to a pig for one passage and was then carried for 8 further passages in rabbits by using infected rabbit blood as transfer material. Aside from a febrile response, no other symptoms were observed in inoculated rabbits.

12 Levaditi, C., Harvier, P., et Nicolau, S., *Annales de l'Institut Pasteur*, 1922, **36**, 107.

13 Ch'en, W. K., PROC. SOC. EXP. BIOL. AND MED., 1933-34, **31**, 1252.

14 Coffey, J. M., *Am. J. Pub. Health*, 1934, **24**, 473.

15 Cox, H. R., *Science*, 1941, **94**, 399.

16 Waddell, M. B., and Taylor, R. M., *Am. J. Trop. Med.*, 1945, **25**, 225.

17 Edwards, J. T., Report of the Imperial Bacteriological Laboratory, Muktesar, India, for the 2 years ending March 1924, p. 32.

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Serial Passage of Hog Cholera Virus in Rabbits.

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Successful adaptation of rinderpest virus of cattle to the rabbit[1] naturally led to similar experiments with hog cholera virus since both of these agents are quite specific for their natural hosts. In the original work on rinderpest alternate transfer of virus from its natural host to the rabbit and then back to the calf was the procedure that made for success. With this alternating technic virulence of the rinderpest virus for the rabbit increased progressively and, following this increase, evidence of disease was found in the rabbit. The results of the experiments on hog cholera virus follow.

Virus Strains Used. Strain A was obtained through the courtesy of Dr. TenBroeck[2] and, as reported by him, it was one used for vaccination in the central portion of the United States. In his work this strain was grown in the presence of fresh minced swine testicle on the chorioallantoic membrane of embryonated eggs for 13 transfers, followed by an equal number of transfers on agar, making 26 transfers in all. From the last transfer lyophilized material was kept from April 5, 1941 until March 24, 1945 when it was injected into a pig. For further preservation spleen from this animal was kept frozen with

1 Baker, J. A., *Am. J. Vet. Research*, 1946, **7**, 179.

2 TenBroeck, C., *J. Exp. Med.*, 1941, **74**, 427.

TABLE I.
Effect on Swine of Hog Cholera Virus (Strain A) Before and After Serial Passage in the Rabbit.

No. of serial transfers in rabbits	Swine		Results in swine				
	No.	Wt, lb	Incubation period, days	Duration of fever, days	Highest temp., °C	Outcome of illness	Reaction to injection of virulent virus
0	1	37	3	9	41.0	Died	
0	22	27	3	11	41.5	"	
0	29	30	3	10	41.3	"	
0	37	24	3	11	41.8	"	
0	40	21	3	8	41.5	"	
5 (a)*	8	80	3		41.5	Killed	
5 (b)	11	70	3		41.2	"	
10 (b)	13	70	?	0	39.3	Survived	Immune
10 (b)	20	25	?	0	39.7	"	"
10 (a)	27	30	3	4	41.3	"	"
15 (b)	42	32	5	2	40.6	"	"
15 (a)	43	30	4	3	40.6	"	"

* (a) indicates first group of serial passages; (b) second group of serial passages.

dry ice until January 2, 1946 when this work began.

Strain B was obtained through the courtesy of Dr. Shope. This strain was recovered by him from a natural outbreak May 26, 1944 and was subsequently carried through 5 transfers in swine. In interim periods between transfers the virus was kept as infected blood in a refrigerator.

Rabbits Used. The rabbits used were from mongrel stock, largely Polish-Dutch, from 3 to 5 months old weighing 1500 to 2500 g.

Procedure Used. Each of 2 rabbits was inoculated intravenously with 1 cc of a 10% suspension of spleen obtained from an infected pig at the height of illness. The rabbits were killed after 5 days and a portion of spleen from each was used for making another 10% suspension. Two rabbits were given 1 cc each of this suspension and injections of the same suspension were made into swine to determine the amount of virus present. Spleen tissue was also frozen in CO_2 and was available for retest if necessary. Tests for contaminating organisms made on blood agar slants and sealed meat medium showed occasional cocci from a few of the pig spleens. Suspensions of rabbit spleens showed no growth. Temperatures were taken and observations made on the rabbits and swine in the morning and evening. Swine that died were autopsied and the diagnosis was made on the finding of typical lesions. Any animals that survived were reinoculated with at least 100 times the infective dose and a control animal injected with the same virus was kept in the same room until its death. If the surviving animal showed no signs of illness from this test injection and exposure period, it was evident that hog cholera virus had been present in the material used for the first inoculation.

Results. Strain A. Test of the spleen suspension of the first rabbit passage showed that 0.01 g of tissue would infect swine, whereas 0.001 g would not. The spleen suspension of the 2nd and subsequent rabbit passages was not titrated, but 0.1 g contained virus, as shown by swine inoculation on the 2nd, 5th, 10th, and 15th passages. These results were repeated in a similar series using as starting material spleen from a different pig and the composite results are shown in Table I.

As Table I shows, strain A was twice passed serially through rabbits for 15 transfers. There was noted a decrease in virulence for swine, as rabbit spleen suspensions from the 10th and 15th serial passages injected intramuscularly produced fever of short duration as the only sign of illness (Table I and Fig. 1). This was followed by complete immunity to an intramuscular injection of 100 times the amount of fully virulent hog cholera virus that produced death in a control animal kept in the same room. Virus attenuated in this way by serial passage in rabbits may make a practical and inexpensive vaccine.

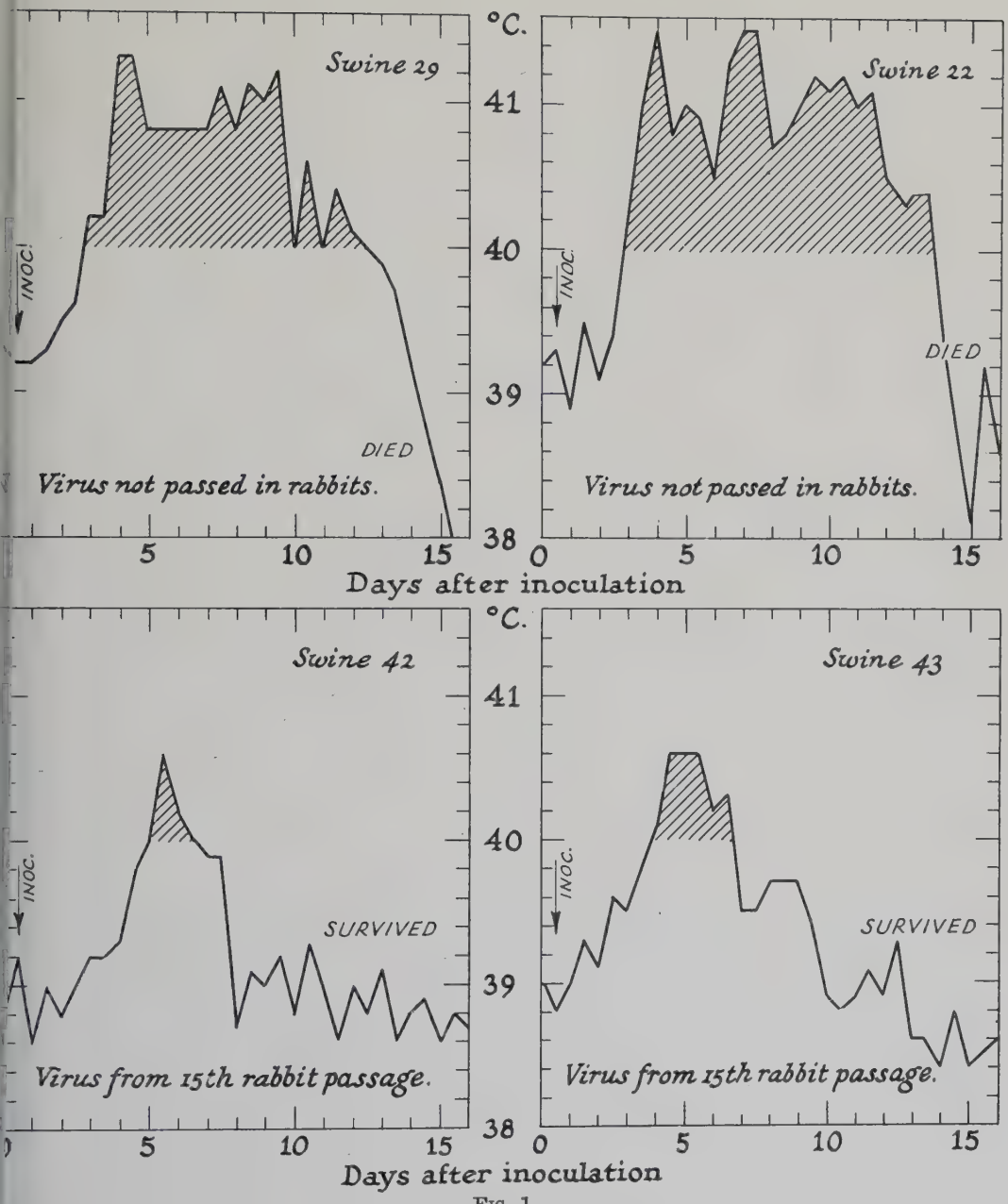

FIG. 1.

Thermal reaction in pigs given hog cholera virus (strain A) not transferred in rabbits and from the 15th serial passage.

Strain B. In the first passage 0.1 g of the rabbit spleen infected pigs but 0.01 g did not. Transfer to a second rabbit failed, since 0.1 g of this spleen did not infect a pig. The alternating technic described briefly above and more fully in the rinderpest paper[1] was then attempted. As Table II shows, swine were infected with 0.1 but not with 0.01 g of spleen from rabbits in the first 3 alternating passages. In no case was the virus detected in the 2nd consecutive rabbit passage. After the 4th and 5th alternation, 0.01 g of

TABLE II.
Hog Cholera Virus (Strain B) Alternated Between Swine and Rabbits.

Alternating passage in swine	Serial passage in rabbits	Test of rabbit spleen for virus by swine injection	
		Amt (g)	Result
1st	1	0.1	+*
		0.01	—
	2	0.1	—
2nd	1	0.1	+
		0.01	—
	2	0.1	—
3rd	1	0.1	+
		0.01	—
	2	0.1	—
4th	1	0.01	+
		0.001	—
	2	0.1	—
5th	1	0.01	+
		0.001	—
	2	0.1	—
6th	1	0.001	+
		0.0001	—
	2	0.1	+
	3	0.1	—
7th	1	0.001	+
		0.0001	—
	2	†	
	3	0.1	—
8th	1	0.001	+
		0.0001	—
	2	†	
	3	0.1	—

* + indicates virus present; —, no virus.
† Not tested.

spleen then infected swine. Again no virus was detected in the 2nd consecutive rabbit passage. After the 6th, 7th, and 8th alternation, 0.001 g then infected swine. With this last increase of virus in the rabbit, transfer was successful for 2 but not 3 serial rabbit passages.

Swine inoculated with infective spleen suspensions from rabbits in the 1st, 2nd, and 3rd alternating passages showed an incubation period of 3 days while a time interval of 4 to 7 days between injections and appearance of fever was noted in those given a similar amount of virus from subsequent alternating passages. It seems evident, therefore, that adaptation of strain B for rabbits was caused by alternating the virus between swine and rabbits.

Effect of Hog Cholera Virus on Rabbits. None of the rabbits inoculated with either strain A or B showed any temperature elevation in early passages. In later passages a slightly increased temperature was noted in some animals inoculated with either strain but temperatures exceeding 40°C seldom occurred. No other signs of illness were noted. None of the animals upon autopsy showed lesions referable to hog cholera.

Discussion. A review by Muir[3] of the divergent results obtained by others in transfers of hog cholera virus to animals other than swine would suggest that some strains are adaptable while others are not. The complete adaptation to rabbits of strain A

[3] Muir, R. O., *J. Comp. Path. and Therap.*, 1940-1943, **53**, 237.

and incomplete adaptation of strain B in our work would support this idea. Also, adaptation of yellow fever by Theiler and Smith,[4] dengue by Sabin and Schlesinger,[5] and perhaps other viruses to animals has not been obtained regularly.

The fact that strain A had been kept under laboratory conditions for a long period and had been cultured outside the body of swine may have influenced its adaptability to the rabbit, although it is possible that strain A was naturally more adaptable. On the other hand, strain B had been isolated recently. It showed poor adaptability and required 6 alternating passages before virus increase occurred in the rabbit spleens and it could be transferred consecutively in the rabbit.

Since neither strain A nor B produces definite signs of illness in rabbits, a study of other strains and perhaps in other laboratory animals should be made especially by the alternating method which, as was shown with strain B and rinderpest, allows opportunity for development of any latent pathogenic potentiality. Such a study may give results similar to those obtained with rinderpest and supply the laboratory animal that has been needed in hog cholera research.

Summary. Two strains of hog cholera virus were inoculated into rabbits. One strain (A) continued in serial passage, became attenuated for swine and, after 15 rabbit passages, produced a febrile reaction of short duration as the only sign of illness. This attenuated strain fully immunized swine to the virulent hog cholera virus. The other strain (B) was present in rabbits for 1 but not 2 consecutive passages. With 6 continued transfers alternately between swine and rabbits, the amount of virus in the rabbit's spleen attained 100 times that found in the initial rabbit transfer. Serial passage in the rabbit was then successful for 2 but not 3 transfers. Evidence of attenuation of this strain was the lengthened incubation period in swine inoculated with the rabbit-passed material. Possible explanations for the difference in adaptability of these strains are discussed.

4 Theiler, M., and Smith, H. H., *J. Exp. Med.*, 1937, **65**, 767.

5 Sabin, A. B., and Schlesinger, R. W., *Science*, 1945, **101**, 640.

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Antihistaminic Substances in Histamine Poisoning and Anaphylaxis of Mice.

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The conditions under which mice can be sensitized and the manifestations of sensitization in mice closely simulate those of anaphylaxis in guinea pigs and other animals (Braun,[1] Schultz and Jordan,[2] Ritz,[3] Sarnowski,[4] Bourden,[5] Weiser, Golub and Hamre[6]). Active sensitizations can be produced with various protein substances, horse, sheep, cow, guinea pig serum, or egg white; passive sensitizations with immune rabbit serum, antihorse guinea pig serum, antihorse rabbit serum, antipneumococcus Type I rabbit serum, etc. The sensitizations are specific and their duration varies from several weeks to several months. Refractoriness occurs after recovery from shock and active desensitization is obtained by

1 Braun, H., *Münch. med. Wschr.*, 1909, **37**, 1880; *Z. Immunf.*, 1910, **4**, 590.

2 Schultz, W. H., and Jordan, H. E., *J. Pharm. and Exp. Therap.*, 1911, **2**, 375.

3 Ritz, H., *Z. Immunf.*, 1911, **9**, 321.

4 Sarnowski, V., *Z. Immunf.*, 1913, **17**, 577.

5 Bourdon, K. L., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 340.

6 Weiser, R. S., Golub, O. J., and Hamre, D. M., *J. Inf. Dis.*, 1941, **68**, 97.

the usual desensitization methods. Specific precipitins are formed in mice actively sensitized against egg white with a titer varying from 1:100 to 1:400.

From all these facts it has been concluded that the "protein shock" in mice is a true anaphylaxis; they fulfill indeed Doerr's[7] criteria of allergy.

The mechanism which ultimately leads to the various anaphylactic manifestations in the different animal species is certainly not the same. The tissue which becomes the principal site of sensitization in guinea pigs is that of the lungs; in rabbits, that of the vascular system; in dogs, that of the liver; etc. The site of sensitization in mice is unknown.

The similarity of the symptoms of anaphylactic shock in guinea pigs or dogs to those of histamine poisoning was first recognized by Dale and Laidlaw.[8] The significant increases of the histamine level in the blood during anaphylactic shock in guinea pigs and dogs are strong support for the "histamine theory" of anaphylaxis. It is today commonly accepted that at least in these animal species the antigen-antibody reaction leads to a liberation of histamine or a histamine-like substance, which is the ultimate cause of the anaphylactic shock. These findings have been interpreted in a more general way, and histamine is considered to be the principal offender in all cases where antigen-antibody reactions in allergy lead to anaphylactic symptoms or to reactions of a similar nature.

However, objections have been raised against such a generalization. For instance, unlike anaphylaxis in guinea pigs and dogs, the histamine blood level decreases in anaphylaxis of rabbits, horses and calves (Code and Hester,[9] Rose and Weil[10]). The connection between allergic manifestations and histamine is especially debatable in mouse anaphylaxis. Whereas the very small dose of 0.6-0.8 mg per kg body weight of intravenously-injected histamine phosphate kills guinea pigs almost instantly, mice are very resistant to histamine, and many animals survive the intravenous injection of 500 mg per kg body weight. In our own experiments, the dose of 750 mg per kg body weight was lethal in all cases. According to these figures, the mouse is about 1,000 times more resistant to histamine than the guinea pig.

The histamine content of the normal mouse averages about 10 mg of histamine per kg body weight. Since mice present the first signs of histamine shock only when 100 mg or more of free base are injected, it is rather improbable that histamine is involved in anaphylactic shock in mice.

In an endeavor to study this question a new approach was used, which was made possible by the recent introduction of the so-called antihistaminic substances supposed to counteract histamine specifically, and considered by many investigators as biologic reagents for histamine. It was of interest to investigate whether histamine poisoning in mice and mouse anaphylaxis responded to these antihistaminic substances in the same way as anaphylaxis of the guinea pig and the dog.

Experimental. I. Influence of Antihistaminic Substances Upon Histamine Poisoning in Mice. Since the toxicity of histamine for mice depends largely upon the concentration of histamine and the rapidity of injection, 2% solutions of histamine phosphate were uniformly used in all experiments; the solutions were injected at the rate of 1 ml per minute. Under these conditions 500 mg of histamine phosphate per kg body weight killed 50% of the mice; 375 mg per kg, 44%. 250 mg per kg body weight occasionally produced a brief period of muscular incoordination and convulsions, but after a few minutes almost all animals regained their normal state.

If antihistaminic substances such as Pyribenzamine* or Benadryl† were given in subcutaneous injections of 10 or 25 mg per kg body weight 15 minutes before the histamine injection, the toxicity of histamine was not

[7] Doerr, R., *Arch. f. Dermat.*, 1926, **151**, 3.

[8] Dale, H., and Laidlaw, P., *J. Physiol.*, 1911, **43**, 182.

[9] Code, C. F., and Hester, H. R., *J. Physiol.*, 1939, **127**, 71.

[10] Rose, B., Braun, H., and Weil, P., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 494.

* Trade name for N′-pyridyl-N′-benzyl-N-dimethyl ethylene diamine monohydrochloride (Ciba).

† Trade name for dimethyl amino ethyl benzhydryl ether hydrochloride (Parke, Davis & Co.).

TABLE I.
Toxicity for Mice of Histamine Phosphate Alone and in Combination with "Antihistaminic Substances."

Histamine doses mg/kg i.v.	No antihistaminic substance	Pyribenzamine		Benadryl	
		10 mg/kg	25 mg/kg	10 mg/kg	25 mg/kg
750	10/10*	3/3		6/6	
500	10/20	11/11	11/11	10/12	14/14
375	10/23	9/11	16/16	11/12	10/10
250	1/13	3/8	11/15	0/5	0/5

Histamine phosphate, 2% solutions, intravenous injection, 1 ml per minute.
Pyribenzamine and Benadryl given 15 minutes before histamine by subcutaneous injection.
* Denotes animals dead/animals tested.

decreased, as is usually the case with guinea pigs or dogs, but, on the contrary, was strongly enhanced. Histamine phosphate alone in a dose of 375 mg per kg body weight was lethal for 10 out of 23 mice (43%). In combination with 25 mg per kg Pyribenzamine, the same dose of 375 mg of histamine killed 16 out of 16 mice (100%) and in combination with 25 mg per kg Benadryl, 10 out of 10 mice (100%). A similar significant increase in toxicity occurred with a combination of 500 mg per kg histamine phosphate with 25 mg per kg Pyribenzamine or Benadryl (from 50% mortality in controls, to 100% with either substance). The dose of 25 mg per kg Pyribenzamine, or Benadryl is nontoxic for mice.

The results (shown in Table I) indicate that the increase in toxicity of histamine is proportional to the dose of Pyribenzamine or Benadryl previously injected. These surprising results indicate that Pyribenzamine or Benadryl, which strongly and specifically counteract and neutralize histamine in guinea pigs and dogs, are definite synergists of histamine in mice.

II. *Influence of Pyribenzamine Upon Active Anaphylaxis in Mice.* The picture of a fully developed mouse anaphylaxis is the following: Five to 10 minutes after the shocking dose is injected, the animals show some excitement, but rapidly become quiet. After a short time, increasing difficulty in breathing develops. This state is followed by loss of coordination, and about 20 minutes later a progressive paralysis of the body starts, beginning with the hind legs. In those instances in which the animal dies, this paralytic state is soon succeeded by a short period of violent convulsions and coma, in which the animals succumb in about half an hour. The entire sequence is sometimes over in 15 minutes, but may last an hour or more. The symptoms are quite similar to those seen in anaphylaxis in guinea pigs, rabbits, or dogs—they merely extend over a much longer period of time.

210 mice were sensitized, in 2 separate groups, by 4 consecutive intraperitoneal injections of 1 ml each of undiluted horse serum every other day. Twenty-one days later a challenging dose of 1 ml of undiluted horse serum, warmed to 37°C, was injected intravenously into 169 mice still alive at that time. Preliminary experiments had shown that this amount, representing in sensitized mice about 2 shocking doses, did not affect normal mice.

Fifty-eight out of the 169 surviving sensitized mice served as controls and received the challenging injection without any other treatment. All of them went into severe shock with a mortality of 89%. The other 111 mice were divided into 4 groups of 20 to 30 animals each and received subcutaneous injections of Pyribenzamine in doses of 10, 25, 30 or 50 mg per kg body weight respectively, 15 minutes before the challenging dose of horse serum was injected intravenously. The challenging injection of horse serum produced in animals, without exception, the depressive stage of shock with varying degrees of prostration. Nevertheless, about one-half of the mice treated with 10 to 30 mg of Pyribenzamine remained free from convulsions and recovered. Thus, the morbidity picture, compared to the controls, was somewhat improved and the mortality rate definitely

TABLE II.
Effect of Pyribenzamine upon Mouse Anaphylaxis.

	Controls No treatment	Treatment with Pyribenzamine (mg/kg) 10	25	30	50
No. of animals dead in shock over No. of animals used	53/58	18/29	13/31	12/24	19/27
% of *deaths in shock*	89%	65%	42%	50%	70%

decreased. In contrast to a mortality rate of 89% for the controls, only 65% of the mice pretreated with 10 mg of Pyribenzamine per kg body weight died (18 out of 29); 42% of those pretreated with 25 mg of Pyribenzamine (13 out of 31); 50% after pretreatment with 30 mg of Pyribenzamine (12 out of 24); and 70% after pretreatment with 50 mg of Pyribenzamine. This last dose of Pyribenzamine is definitely toxic for mice and it is probable that a number of the deaths included in the 70% are due to Pyribenzamine. (LD_{50} of Pyribenzamine by subcutaneous injection for mice is 75 mg per kg body weight). Table II shows the results obtained.

It is significant that in no case was complete freedom of all shock symptoms ever obtained, even with high doses of Pyribenzamine, although significant protection against convulsions and death was afforded. In any event, protection conferred on the sensitized mice was much less spectacular, as compared to the certainly more complete protection obtained in sensitized guinea pigs with doses of Pyribenzamine only 1/50 as great.

Discussion. It has often been suggested that a positive correlation exists between a species sensitivity to histamine and the ease with which one may induce anaphylaxis in this species. Indeed, guinea pigs, dogs, rabbits, or man which are highly sensitive to histamine, become sensitized quite easily. In these same species, Pyribenzamine and other antihistaminic substances have been shown to be most powerful antagonists of histamine, as well as of anaphylaxis (Dekanski,[11] Mayer, Huttrer and Scholz,[12] Mayer,[13] Yonkman, Hays and Rennick,[14] Arbesman, Koepf and Miller[15]).

In vitro, small doses of Pyribenzamine prevent several typical histamine effects on isolated guinea pig intestines, uterus, or lungs; *in vivo,* 5 mg per kg protect guinea pigs against 100 and more lethal doses of histamine. Anaphylactic shock in guinea pigs can be prevented by doses as small as 0.1 to 0.5 mg per kg body weight; somewhat higher doses are active against histamine poisoning or anaphylactic shock of dogs (3-3.5 mg per kg body weight).

Contrary to guinea pigs and dogs, the mouse is highly resistant to histamine; it tolerates about 1,000 times more histamine than the guinea pig. Correspondingly, the mouse is very resistant to any sensitizing procedures.

A similar difference existed in the response to antihistaminic substances. Pyribenzamine and Benadryl not only failed to protect mice from histamine poisoning, but definitely acted as synergists to histamine. After an injection of 25 mg per kg body weight of Pyribenzamine, for instance, the toxicity of histamine in mice was doubled.

That the antihistaminic substances act quite differently in mice than they do in guinea pigs seems not to be a fortuitous occurrence, but rather seems to be in agreement with the theory that antihistaminic substances are competitors of histamine.

An analysis of the general and quite uncharacteristic picture of acute histamine poisoning in mice does not give any answer to the question of whether histamine acts in mice in the same manner, that is, on the same organs and by identical mechanisms as in guinea pigs and dogs. The absence of any protection by antihistaminic substances in the

[11] Dekanski, J., *J. Physiol.,* 1945, **104**, 151.

[12] Mayer, R. L., Huttrer, C. P., and Scholz, C. R., *Science,* 1945, **102**, 93; *Fed. Proc.,* 1945, **4**, 129.

[13] Mayer, R. L., *J. Allergy,* 1946, **17**, 153.

[14] Yonkman, F. F., Hays, H. W., and Rennick, B., *Fed. Proc.,* 1945, **4**, 144.

[15] Arbesman, C. E., Koepf, G. F., and Miller, G. E., *J. Allergy,* 1946, **17, 203.**

case of histamine poisoning of mice seems to indicate that different mechanisms are involved. On the other hand, the synergism of histamine and "antihistaminic" substances cannot be explained by a possible histamine-like activity of these "antihistaminic substances." There is no such action in mice. We rather conclude that in the mouse, histamine affects organ systems other than those affected in histamine-sensitive animals, and that in these points of attack, antihistaminic substances are unable to compete with histamine. On the contrary, each poison acts on its own, both toxicities being additive in effect.

It seems quite inconsistent with the synergistic action of histamine and Pyribenzamine that the latter should have a certain protective action in mouse anaphylaxis. The very fact that Pyribenzamine in the mouse does not act as an antihistaminic agent, leads to the belief that this "protection" against mice anaphylaxis is not due to a specific antianaphylactic effect, comparable to that which Pyribenzamine exercises in anaphylaxis of guinea pigs or dogs. It is rather probable that some pharmacologic properties of Pyribenzamine other than its antihistaminic power are responsible for this protection. It may be that it acts here through its local anesthetic power, since it is known that general anesthetics, as well as local anesthetics, have a definite influence upon anaphylaxis (Wolfsohn[16]). The same problem has been discussed in a study concerning the influence of Pyribenzamine upon contact dermatitis in guinea pigs (Mayer[17]). Other experiments by Yonkman, Hays, Chess and Rennick[18] seem to indicate that the general pharmacological activity of an antihistaminic agent such as Pyribenzamine may well include other properties than those associated with antihistaminic power.

Further experiments are necessary to correlate allergy in mice with that of other animals and to identify the "anaphylactic poison" of mouse anaphylaxis, which quite probably is not histamine.

Conclusions. (1) "Antihistaminic substances" act as synergists in the histamine poisoning of the mouse; (2) contrary to the action on histamine poisoning, a certain protective power in mouse anaphylaxis has been observed; (3) the possible conclusions following from these observations are discussed.

16 Wolfsohn, G., *The Palestine and Near East Med. J.*, 1944, **3**, 11.

17 Mayer, R. L., *Ann. Allergy*, in press.

18 Yonkman, F. F., Hays, H. W., Chess, D., and Rennick, B., *J. Pharm. and Exp. Therap.*, in press.

15543

Concentration and Properties of the Adrenocorticotrophic Substance in Female Human Urine.

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Adrenocorticotrophin has been demonstrated to be present in the blood serum[1] and in the anterior pituitary gland. Its possible presence in the urine has been suggested by the effects of injecting urine from pregnant[2] and nonpregnant[3] women on the adrenal cortex of young guinea pigs. No attempt has been made to identify the substance which may have caused the adrenal hyperplasia. Since the corpus luteum hormone[3] and estrogens[4,5] can induce hyperplasia of the

1 Golla, M. L., and Reiss, M., *J. Endocrinology*, 1942, **3**, 5.

2 de Boissezon, P., *Bull. histol. appliq. physiol.*, 1936, **13**, 129.

3 Blumenthal, H. T., *J. Lab. Clin. Med.*, 1945, **30**, 428.

4 Janes, R. G., and Nelson, W. O., *Am. J. Physiol.*, 1942, **136**, 136.

5 Uotilla, U. Y., *Endocrinology*, 1940, **26**, 123.

TABLE I.
The Effect of Female Urine and Fractions Thereof on the Hypertrophy of Young Male Rat Adrenals.

Exp. No.	No. of rats	Starting wt of rats g	Material tested	Adrenal wt, mg	Mg adrenal per 100 g body wt
I	12	50.9	*Fresh whole female urine*—0.5 cc intraperitoneally, twice daily for 4 days	19.5	36.1
	5	49.6	*Dialysate of above urine*—Administered as above	19.8	35.6
	12	50.8	*Control*—isotonic saline as above	11.2	17.8
II	7	40.7	*Conc. whole urine* (2.5:1)—0.5 cc intraperitoneally, twice daily for 4 days	12.6	24.5
	7	40.7	*Acetone ppt. of dialyzed urine* (Prep. W-1)—6 mg daily, administered as above	14.6	29.3
	7	40.9	*Control*—isotonic saline (pH 7.7)	8.0	15.4
III	7	37.3	*Conc. whole urine*—0.5 cc intraperitoneally, twice daily for 4 days	10.8	27.1
	7	39.0	*Acetone ppt. of dialyzed urine* (Prep. W-2)—6 mg daily, administered as above	14.6	31.1
	6	38.3	*Control*—isotonic saline (pH 7.7)	8.4	17.3

adrenal cortex, the possibility that the cortical hyperplasia might be due to the sex hormones found in the urine has not been eliminated.

In this paper, we present evidence that the adrenocorticotrophic activity of human urine does not depend on the ovarian hormones and that it is probably due to a protein which may be the excretory form of pituitary adrenocorticotrophin.

Experimental. Freshly voided human female urine, dialyzed urine and precipitated urinary proteins were tested for adrenocorticotrophic activity in white rats by the methods of Moon[6] and Sayers, *et al.*[7,8] for pituitary adrenocorticotrophins.

Young male rats (23-30 days old) were injected intraperitoneally twice daily for 4 days, with 0.5 cc portions of urine which had been voided and filtered within the previous hour. The urine was obtained by pooling the output of 5 nonpregnant nonmenstruating young women. The control rats received isotonic saline solution at the same time. After the experimental period, the rats were sacrificed and the adrenals removed and weighed. The results of this experiment (I) are shown in Table I. The greater adrenal weight of the experimental rats over that of the controls indicates the presence of adrenocorticotrophic activity in the urine, confirming previous observations.[3]

A sample of urine was dialyzed for 24 hours at 0° to remove the greatest portion of the estrogens present. When the dialyzed urine was tested as in the above experiment, similar results were obtained (Table I, Exp. I). This experiment indicates that the urinary estrogens are not the causative agents for the adrenal hypertrophy.

In order to have a standard supply of urinary adrenocorticotrophin on hand, a concentrate of female urine was made in the following manner. Freshly voided urine was filtered and dialyzed with agitation against 20 volumes of distilled water at 0° for 24 hours. The dialysis was repeated with another 20 volumes of fresh distilled water. The dialyzed urine was then concentrated to approximately 1/5 volume by pervaporation under toluene. To the concentrated urine 5-6 volumes of acetone were added and the mixture kept in the cold room for 48 hours. The precipitate was centrifuged, washed twice with acetone and dried over P_2O_5. A pooled sample of 2.5 l. of urine yielded 505 mg of a light gray powder which gave a strong biuret test and contained 7.34% nitrogen (microkjeldahl, Prep. W-1). When another 1 liter sample of urine was put through this

[6] Moon, H. D., PROC. SOC. EXP. BIOL. AND MED., 1937, **35**, 649.

[7] Sayers, G. M., Liang, T. Y., and Long, C. N. H., *Endocrinology*, 1946, **38**, 1.

[8] Sayer, M., and Sayers, G., *Proc. Fed. Am. Soc. Exp. Biol.*, 1946, **5**, 200.

TABLE II.
Effect of Female Urine and Fractions Thereof on Adrenal Ascorbic Acid of Young Male Rats.

No. of rats	Rat wt, g	Material tested	Ascorbic acid per adrenal μg	Mg ascorbic acid per 100 g adrenal
5	40.2	Fresh whole urine—2.0 cc	16.9	277
5	44.6	Acetone ppt. of dialyzed urine (Prep. W-1); 6 mg in 2.0 cc isotonic saline-phosphate buffer (pH 8.0)	15.5	267
5	36.4	Prep. W-1 heated at 100° for 30 min; 6 mg in 2.0 cc isotonic saline-phosphate buffer (pH 8.0)	45.1	490
4	35.0	Crystalline egg albumin—6 mg in 2.0 cc isotonic-saline-phosphate buffer (pH 8.0)	32.6	473
14	39.5	Control—4 mg gelatin in 2.0 cc isotonic saline-phosphate buffer (pH 8.0)	32.6	501

procedure, a yield of only 52 mg of powder was obtained (Prep. W-2).

A solution of each of the above preparations in isotonic saline-0.01 M phosphate buffer (pH = 7.7; 4 mg per cc) was tested in the manner previously described. The results (Table I, Exp. II and III) show that both preparations have adrenocorticotrophic activity. These experiments are a further demonstration that the urinary steroid hormones do not cause the observed adrenal hypertrophy. Dialysis and the acetone treatment can be expected to remove all but traces of urinary steroids.

The method of Sayers, *et al.*[7,8] depends on the fact that pituitary adrenocorticotrophin will cause a rapid and profound decrease in the ascorbic acid level of the adrenals. The method was modified in the following manner to test for the presence of adrenocorticotrophin in urine. Young male rats were anesthetized with nembutal and 2.0 cc of the solution to be tested were injected intraperitoneally. After 1.5 hours, the animals were sacrificed, the adrenals removed and the ascorbic acid content determined. The ascorbic acid was measured by the method of Carruthers.[9] In our hands, this method was found satisfactory in that known amounts of ascorbic acid could be estimated with an error of less than 7%.

Fresh female urine and Prep. W-1, when tested in the above manner, showed a marked effect in depressing the adrenal ascorbic acid as compared to the controls (Table II). The control animals received 4 mg of gelatin in 2 cc of isotonic saline-phosphate buffer (pH = 8.0). The gelatin was used as a foreign-protein control. When a solution of Prep. W-1 was heated in a water-bath at 100° for 30 minutes, it no longer possessed any depressing effects on the adrenal ascorbic acid level. Since Prep. W-1 is also nondialyzable, this would seem to indicate that the active material is protein in nature. That nonspecific antigenic proteins are probably ineffective in this test was shown when egg-albumin (3X recrystallized) was assayed and found to have no effect on the adrenal ascorbic acid level.

A solution of Prep. W-1 was also tested on hypophysectomized rats. One of the adrenals of the hypophysectomized animals was removed and a solution of 6 mg of Prep. W-1 in isotonic saline-phosphate buffer was injected. After 1.5 hours the second adrenal was removed and the ascorbic acid level in each determined. A depression in adrenal ascorbic acid (145 mg/100 g adrenal tissue) was found. This indicates that the urinary adrenocorticotrophin acts in the same manner as the pituitary adrenocorticotrophins in that both decrease the ascorbic acid level of the adrenals markedly in a relatively short time. Further, the urinary adrenocorticotrophin acts without the mediation of the pituitary.

It can, therefore, be seen that the urinary and pituitary adrenocorticotrophins are qualitatively similar in their effects. This establishes the possibility that they are identical, or, at least, closely related, and that the urinary adrenocorticotrophin is an excretory form of the pituitary adrenocorticotrophin.

[9] Carruthers, A., *Ind. Eng. Chem., Anal. Ed.*, 1942, **14**, 826.

The determination of the presence of adrenocorticotrophin in male urine and the identification of the adrenocorticotrophic protein in female urine are now under investigation.

Summary. Normal female urine contains adrenocorticotrophic activity which has been shown not to be due to its estrogen content. Being nondialyzable and thermolabile, the active material appears to be a protein. It is similar to pituitary adrenocorticotrophin in its ability to produce adrenal weight increases and to depress the adrenal ascorbic acid level in normal and hypophysectomized male rats.

15544

Effect of Boric Acid on Avian Malaria.

A. O. SEELER AND C. MALANGA. (Introduced by H. Molitor.)

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The recent paper by Hardcastle and Foster[1] reporting encouraging results with the use of borax in the control of cecal coccidiosis in poultry suggested the possibility that boron derivatives might be effective as chemotherapeutic agents in certain types of avian malaria.

Trophozoite-induced *Plasmodium gallinaceum* infections were established in S.C. White Leghorn chicks weighing 50 g by intravenous inoculation of 200,000,000 parasitized erythrocytes per kg; trophozoite-induced *P. cathemerium* infections were induced in Peking ducklings weighing 50 g by the intravenous injection of 500,000,000 parasitized erythrocytes per kg. Sporozoite-induced *P. gallinaceum* infections were used in prophylactic tests and were established in chicks of 50 g weight by injecting intravenously 0.2 cc per chick of a suspension of sporozoites prepared by grinding 100 infected mosquitoes in 20 cc of chicken plasma.

[1] Hardcastle, A. B., and Foster, A. O., *Proc. Helminthological Soc. of Washington*, 1944, **11**, 60.

TABLE I.
Effect of Boric Acid on Trophozoite and Sporozoite Induced Avian Malaria Infections.

No. birds	Drug	Dose	Average % erythrocytes parasitized at peak of infection
	I. Trophozoite-induced *P. cathemerium* infections in ducklings.		
4	Boric acid	2.0% in diet	1.3
4	" "	1.0 " " "	6.6
3	Quinine	80 mg/kg p.o.	5.6
3	"	40 " "	3.1
6	Controls	—	25.6
	II. Trophozoite-induced *P. gallinaceum* infections in chicks.		
5	Boric acid	2.0 % in diet	2.4
5	" "	1.0 " " "	31.9
3	Sulfadiazine	0.05 " " "	<0.1
3	"	0.025 " " "	0.5
6	Controls	—	66.1
	III. Sporozoite-induced *P. gallinaceum* infections in chicks.		
4	Boric acid	2.0% in diet	0.01*
4	" "	1.0 " " "	1.7
4	Sulfadiazine	0.1 " " "	0.00
4	Controls	—	30.0
4	Quinine	0.4 " " "	22.0
4	Atabrine	0.4 " " "	19.7
4	Sulfadiazine	0.2 " " "	0.00
8	Controls	—	17.6

* One chick died, two showed zero counts.

The boric acid was administered by incorporating it in the diet, a commercial chick starting mash, in the amounts shown in Table I. The birds were given the drug-containing diet immediately after inoculation and were permitted to feed *ad libitum* throughout the period of the test. The trophozoite-infected birds were continued on the drug-diet for 5 days. The chicks receiving the sporozoite inoculation were fed the drug-diet for 3 days and were then transferred to the regular untreated stock diet for an additional 6 days.

At the end of the respective test period, thin blood smears were prepared, stained with Giemsa, and the effect of the therapy was judged by determining the number of parasitized cells among 10,000 erythrocytes. In each experiment a group of untreated birds and at least one group treated with a drug of known antimalarial activity served as controls.

The results of the experiments on trophozoite- and sporozoite-induced avian malaria are given in Table I. Whereas quinine, atabrine and sulfadiazine can be used interchangeably as suppressives for the trophozoite infections, only sulfadiazine has prophylactic activity against *P. gallinaceum*. It is apparent that boric acid at relatively high concentrations in the diet had a marked suppressive effect on both *P. cathemerium* and *P. gallinaceum* infections. Boric acid at high doses also showed definite prophylactic activity against *P. gallinaceum* infections.

Thus boric acid, unlike quinine and atabrine, possesses both suppressive and prophylactic activity. It should be noted, however, that the amounts of boric acid required to produce an effect on avian malaria closely approach toxic levels.

15545

Antifibrillating Action of N-Methyl-Dibenzyl-Amine and Some of Its Derivatives.

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Quinidine is the best known among the drugs effective against cardiac fibrillation induced by electrical stimulation or other agents. Other drugs have been reported, some with chemical structure resembling that of quinidine, and others entirely different, which also show some antifibrillating action.

We have found that α-fagarine, (an alkaloid isolated by Stuckert[1] from *Fagara coco* (collected by Gill Engler) exerts an antifibrillating effect as potent as, or more potent than, quinidine itself. It counteracts the influence of several fibrillating agents (faradic currents,[2,3] coronary occlusion[4]) and causes recovery of the sinus rhythm in cases of accidental or spontaneous auricular fibrillation[5] in animal experimentation or in man.[6,7]

Deulofeu and Labriola[6] have established that α-fagarine is a tertiary base to which the following tentative formula can be assigned:

1 Stuckert, G., *Investigaciones de laboratorio de Quimica Biológica*, Córdoba, Vol. I, 1933; Vol. II, 1938.

2 Moisset de Espanés, E., *Rev. Soc. Arg. Biol.*, 1937, **13**, 112; *C. R. Soc. Biol.*, 1938, **127**, 118.

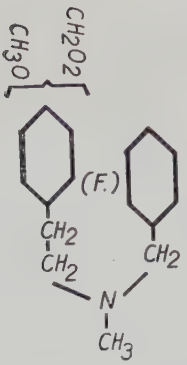
3 Martínez, C., *Medicina* (Buenos Aires), 1944, **4**, 109.

4 Moisset de Espanés, E., *Rev. Soc. Arg. Biol.*, 1937, **13**, 116; *C. R. Soc. Biol.*, 1938, **127**, 233.

5 Moisset de Espanés, E., *Amér. Clín.*, 1945, **8**, 41; Moisset de Espanés, E., y Moyano Navarro, B., *Rev. Soc. Arg. Biol.*, 1936, **12**, 137; *C. R. Soc. Biol.*, 1938, **127**, 510.

6 Deulofeu, V., Labriola, R., Orías, O., Moisset de Espanés, E., y Taquini, A., *Science*, 1945, **102**, 69; *Ciencia e Investigación*, 1945, **1**, 527.

7 Taquini, A., *Rev. Arg. Cardiol.*, 1945, **12**, 83.



The possibility of preparing easily a series of bases derived from methyl-dibenzyl-amine, which has a chemical structure greatly resembling that of the possible structure of α-fagarine, and the hope of finding new antifibrillating substances which, because of efficiency or facility of preparation, would offer advantages, induced us to assay them pharmacologically. Methyl-dibenzyl-amine, which also exerts some antifibrillating effect, as shown by Martinez,[3] was taken as standard to compare the activity of its derivatives.

So far we have studied the following bases, arranged according to the increasing complexity of the substituents attached to the aromatic nuclei: (1) N-methyl-dibenzyl-amine; (2) N-methyl-o-anisyl-p-anisyl-amine; (3) N-methyl-di(o-anisyl)-amine; (4) N-methyl-di-(3,4-dimethoxy-benzyl)-amine; (5) N-methyl-di-(2,3-dimethoxy-benzyl) - amine; (6) N-methyl-di-(p-anisyl)-amine; (7) N-methyl - benzyl - piperonyl - amine; (8) N-methyl-di-(piperonyl)-amine; (9) N-methyl-p-anisyl-piperonyl-amine. The following bases were also studied: (10) N-ethyl-dibenzyl-amine; (11) N-methyl-benzyl-anisidine; (12) N-methyl-benzyl-phenethyl-amine. We shall refer to the above mentioned bases by the number preceding each. (Fig. 1).

All the bases were prepared under the direction of Doctors V. Deulofeu and R. Labriola in the Laboratory of Organic Chemistry in the Facultad de Ciencias Exactas, Fisicas y Naturales de Buenos Aires. Some are already known and the others were obtained by classical methods. The description of the new bases will be given elsewhere.

Rabbits weighing from 1200 to 1800 g were anesthetized with 0.6 g of dial per kg of body weight, half of that dosage being injected intravenously and the other half intraperitoneally. The heart was exposed, with the pleural sacs left untouched, and the thresholds to obtain both auricular and ventricular fibrillation with faradic current were determined. Control experiments showed that such thresholds do not show appreciable variations within the 2 or 3 hours following the beginning of anesthesia.

Two, 7, 17, and 32 minutes after intravenous injection of the substance to be tested, the threshold was again determined, and the maximal variation was taken as indicative of the activity of the drug for comparative purposes.

Table I summarizes the results obtained. The activity of each drug is expressed according to the percentage of threshold increase found after injection. Each figure is the average of the values found in the different animals injected with the same drug. The relative value represents the ratio of the activity of the investigated substance to that of the same dose of the standard substance (methyl-dibenzyl-amine), to which was assigned an activity value of 100. The opponent action of both quinidine and α-fagarine against auricular and ventricular fibrillation was also determined for comparative purposes.

The results show that methyl-dibenzyl-amine exerts antifibrillatory action and that its potency is about the same on auricular and ventricular fibrillation (Table I). The addition of either methoxylic or dioxymethylenic groups to the aromatic nuclei of methyl-dibenzyl-amine, obviously modified its effect. In some cases it decreased (bases 4 and 5) and in some others it increased (bases 3 and 9) but the change did not affect to the same extent the auricular and ventricular effects. All the bases to which only methoxylic groups were added (bases 2, 3, 4, 5, and 6) showed increased activity against ventricular fibrillation, whereas those to which only dioxymethylenic groups were added, (bases 7 and 8), showed increased activity against auricular fibrillation as compared with that of the original nucleus. Bases either having no such groups (bases 1, 10, 11, and 12) or hav-

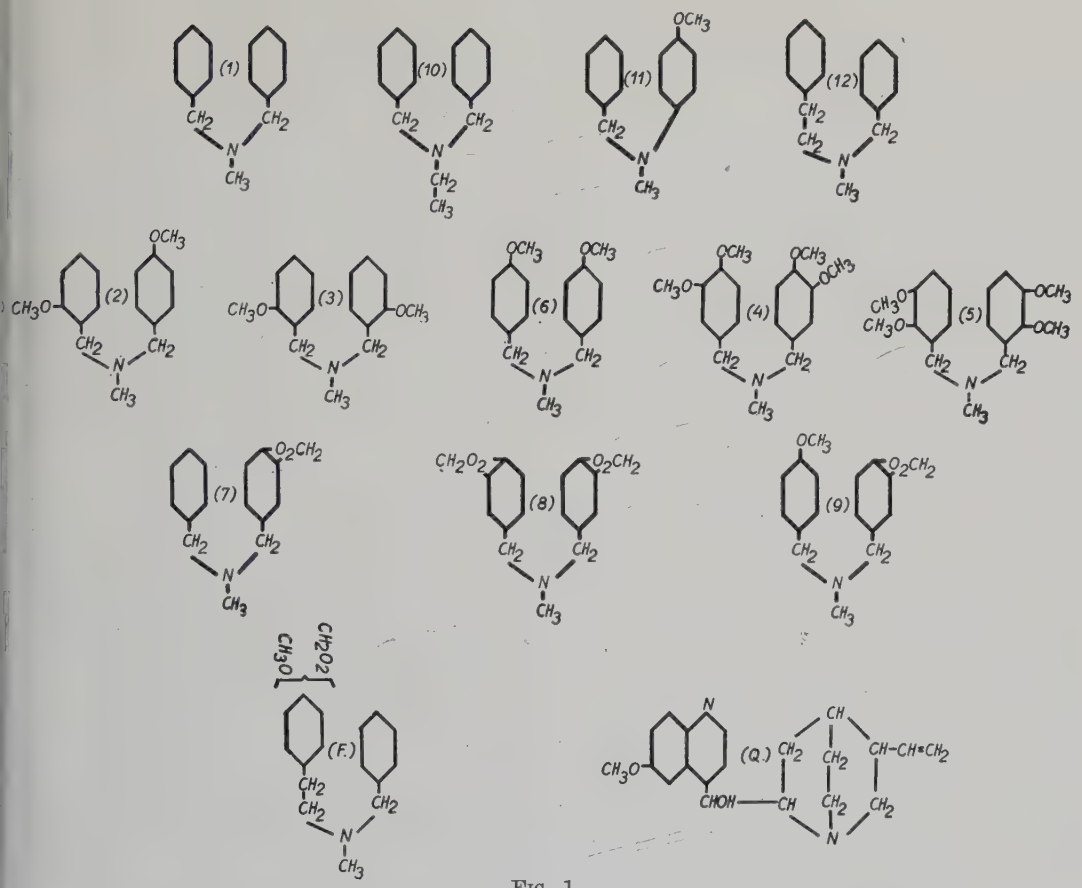


FIG. 1.

(1) N-methyl-dibenzyl-amine; (2) N-methyl-o-anisyl-p-anisyl-amine; (3) N-methyl-di(o-anisyl)-amine; (4) N-methyl-di-(3,4-dimethoxy-benzyl)-amine; (5) N-methyl-di-(2,3-dimethoxy-benzyl)-amine; (6) N-methyl-di-(p-anisyl)-amine; (7) N-methyl-benzyl-piperonyl-amine; (8) N-methyl-di-(piperonyl)-amine; (9) N-methyl-p-anisyl-piperonyl-amine; (10) N-ethyl-dibenzyl-amine; (11) N-methyl-benzyl-anisidine; (12) N-methyl-benzyl-phenetyl-amine.

ing both (one of each) (base 9) showed practically the same degree of activity against both auricular and ventricular fibrillation.

Ethyl-dibenzyl-amine (base 10) also was of interest because substances mentioned in the literature as having antifibrillatory activity have ethyl groups attached to nitrogen. In our substance the substitution of the ethyl for the methyl group rather reduced the activity of the standard substance.

Methyl-benzyl-phenetyl-amine (base 12) with a chemical structure even more closely resembling the proposed structure for α-fagarine than methyl-dibenzyl-amine, our standard substance, showed a somewhat higher activity than the latter.

Furthermore, outside the methyl-dibenzyl-amine series, the aromatic base methyl-benzyl-anisidine was investigated, and showed a markedly weak potency against both auricular and ventricular fibrillation, indicating that the difference in chemical structure produced a difference in pharmacological properties.

Among the substances with higher potency than our standard substance, base 2 (methyl-o-anisyl-p-anisyl-amine) showed a very transient effect (it vanished in approximately 5 minutes) and base 3 (methyl-di(oanisyl)-amine) was highly toxic, producing an intense convulsive attack, more tonic than clonic, the intensity in proportion to the administered dose, appearing sometimes before

TABLE I.

Table Showing the Increase of Faradic Threshold to Fibrillation in Per cent of Initial Value and the Potency of Its Activity as Compared with That of Methyl-dibenzyl-amine at the Same Doses.

		Auricles			Ventricles		
Drug	Dose, mg/kg	No. of animals	% of fibrillation threshold increase (avg and P.E.)	Relative activity	No. of animals	% of fibrillation threshold increase (avg and P.E.)	Relative activity
1	1	24	18.3 ± 1.11	100	20	18.4 ± 1.01	100
	5	29	27.1 ± 2.15	100	20	32.8 ± 2.37	100
2	1	10	18.5 ± 6.65	101	10	25.7 ± 6.74	140
3	½	8	25.0 ± 6.13	137	8	34.6 ± 4.12	188
4	1	11	13.3 ± 1.23	73	11	16.1 ± 2.99	87
5	1	12	10.0 ± 5.81	55	12	14.4 ± 4.32	78
6	1	12	10.2 ± 3.24	56	11	29.4 ± 2.21	160
7	5	10	31.0 ± 4.07	114	10	18.8 ± 6.87	57
8	5	9	29.2 ± 1.98	108	10	12.4 ± 3.81	38
9	1	12	20.3 ± 2.36	111	12	23.3 ± 1.48	127
10	5	10	22.7 ± 1.67	84	10	23.9 ± 3.78	73
11	5	12	1.8 ± 3.84	7	11	5.2 ± 1.36	16
12	1	11	22.3 ± 2.06	122	11	21.6 ± 2.02	117
Q	1	17	21.8 ± 2.87	119	12	16.8 ± 2.60	91
F	1	21	24.8 ± 2.55	135	12	30.3 ± 2.12	165

the injection ended and ceasing under new doses of "dial."

Even though quinidine showed some higher activity against auricular fibrillation, its potency was of the same order as that of our standard substance; α-fagarine, on the other hand, being perhaps more effective against ventricular than against auricular fibrillation, showed, in general, a higher potency than methyl-dibenzyl-amine.

It seems necessary to emphasize that a strict ratio does not exist between the increase in potency as compared with the increase of the administered dose, as generally occurs in pharmacology. Also the relation dose/effect is different for each substance of the series; therefore, the relative activity may not be the same if doses other than those we have used are compared. Furthermore the toxicity of one substance as compared with the others does not necessarily parallel antifibrillating activity. More study will be needed to indicate which one is the most convenient from the practical point of view.

Summary. The antifibrillating activity of a series of tertiary bases with a carbon skeleton related to the possible chemical structure of α-fagarine has been assayed in rabbits. Eight of them were derivatives of methyl-dibenzyl-amine by addition of either methoxylic or dioxomethylenic groups to the aromatic nuclei; the others, also chemically related, were ethyl-dibenzyl-amine, methyl-benzyl-anisidine and methyl-benzyl-phenetyl-amine. Their effects were compared with those of quinidine and α-fagarine, tested under the same conditions.

All of them showed antifibrillating activity. The maximal effect, however, was given by α-fagarine. The addition of methoxylic and dioximethylenic groups modified the activity of methyl-dibenzyl-amine, the former enhancing the effect against fibrillation of the ventricles and the latter enhancing the effect against auricular fibrillation.

15546

Plasma Levels after Repository Injections of Penicillin in Water-in-Oil Emulsions.

H. William Harris, Clare Wilcox, and Maxwell Finland

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A comparison of the penicillin levels obtained in the same patients after the same intramuscular dose of penicillin given in saline and in a water-in-oil emulsion as advocated by Freund and Thomson[1] was presented in a previous communication.[2] Some variations were noted in the maximum concentrations obtained and considerably greater differences were noted in the maintenance of levels in the blood after any given dose. Intramuscular doses of 100,000, 200,000 and 300,000 units were given. Higher and better sustained levels followed the larger doses. There was no constant or striking difference between the results obtained in the same subjects after the same dose given in the 2 vehicles. A dose of 300,000 units in a beeswax-peanut oil mixture gave maximum concentrations which were appreciably lower, but the levels were much better sustained than with the same dose given in saline or in the water-in-oil emulsion. The emulsion base used in the previous study was Pendil, a "chloresterol-derivative and peanut oil" as described by Freund and Thomson.

A second series of observations are reported here with the use of a similar preparation, "Emulgen,"* which is composed of "sesame oil and cholestrin base." Chlorobutanol 3% is added as a preservative and local anesthetic. The methods used were the same as in the previous studies. Each subject was given a single dose of 200,000 or 300,000 units in saline or in the water-in-oil emulsion and

1 Freund, J., and Thomson, K. J., *Science*, 1945, **101**, 468.

2 Ory, E. M., Wilcox, C., and Finland, M., Proc. Soc. Exp. Biol. and Med., 1946, **62**, 86.

* Supplied by Dr. C. O. Miller, Lakeside Laboratories, Inc.

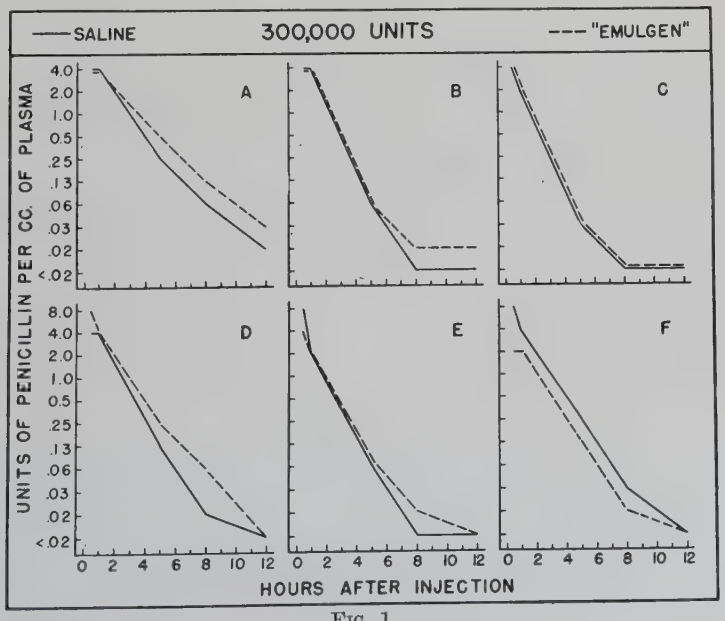

Fig. 1.

Plasma penicillin levels in 6 subjects after intramuscular injections of 300,000 units in saline and in a water-in-oil emulsion.

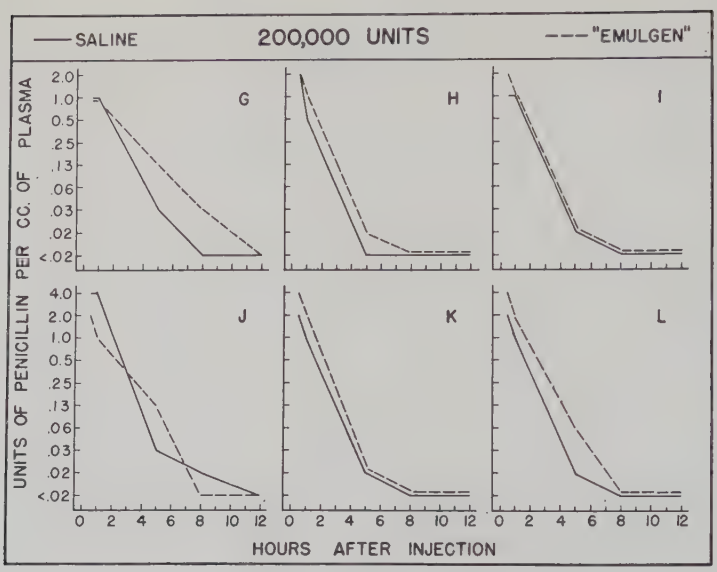

FIG. 2.
Plasma penicillin levels in 6 subjects after intramuscular injections of 200,000 units in saline and in a water-in-oil emulsion.

TABLE I.
Average Concentrations of Penicillin in Plasma Following Intramuscular Injections in Two Vehicles.

Hours after injection	Units per cc of plasma			
	After 200,000 units in		After 300,000 units in	
	Saline	"Emulgen"	Saline	"Emulgen"
½	2.00	2.50	5.33	4.33
1	1.42	1.33	3.33	3.00
5	0.016	0.06	0.13	0.17
8	0.003	0.005	0.02	0.04
12	0	0	0.003	0.008

the same dose was given to the same subjects 2 or 3 days later in the same final volume of the alternate vehicle. A single batch of penicillin was used in this study. This was a purified product which consisted almost entirely of crystalline penicillin G.† Special care was taken to obtain a smooth, creamy emulsion as advocated by Freund and Thomson. There was no local irritation from any of the injections. Blood for penicillin levels was drawn at ½, 1, 5, 8 and 12 hours after the injections. The results are shown graphically for each subject in Fig. 1 and 2 and the average levels for each dose level are given in Table I. Comparable, but not the same subjects were used for the 2 dosages.

As in the previous report, the maximum concentrations varied somewhat in different subjects, but there were greater variations in the rate of decline in the plasma levels. After a dose of 300,000 units, demonstrable levels were no longer present at 12 hours except in one subject. After 200,000 units, there was no demonstrable penicillin in the plasma after 8 hours in almost every instance. In about one-half of the subjects receiving each dose, the penicillin levels were slightly better sustained when given in water-in-oil emulsion. The average blood levels after 5 and 8 hours were also somewhat higher in subjects receiving the emulsion. Much more striking, however, is the fact that the plasma levels obtained after 300,000 units in saline

† Supplied by the Commercial Solvents Corporation.

were considerably higher and much better sustained than those observed after 200,000 units given in the same volume of the water-in-oil emulsion.

Conclusion. The previous observation that only a slight and inconstant prolongation of penicillin blood levels is obtained by the use of a water-in-oil emulsion is confirmed. The present findings suggest further that a substantial increase in the intramuscular dose of penicillin in the same volume of saline will give considerably higher and better sustained plasma levels than the smaller dose given in a water-in-oil emulsion and the disadvantages of the latter method are thus avoided.

15547

A Constant Current Square Wave Stimulator.

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In the study of neuromuscular excitability, both experimental and clinical, a source of stimulating electric current is required. As the tests employed have been refined, there has been simultaneous refinement and complication of the apparatus required to perform them, with the development of a multiplicity of stimulators, each designed for one specific test. It is the purpose of this paper to describe the construction of a stimulator which may be used both for the older and the more recent tests, yet which is not unduly complex, and which may be constructed from standard radio components.

This stimulator is capable of producing waves of essentially square form as seen in Fig. 1, with constant current at full output of 20 milliamperes so long as the output re-

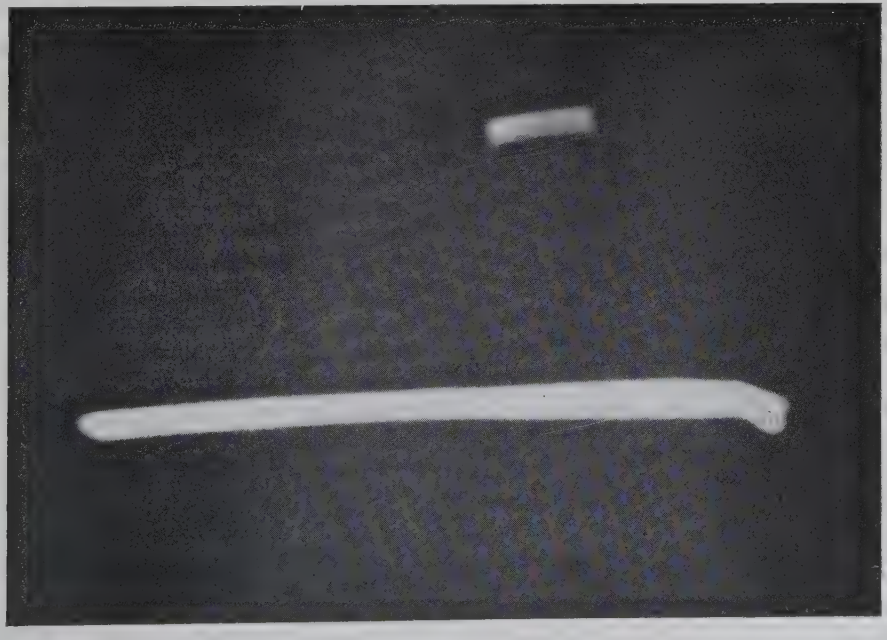

Fig. 1.

Cathode ray oscillographic tracing of stimulus of 5 milliseconds duration, showing square wave form.

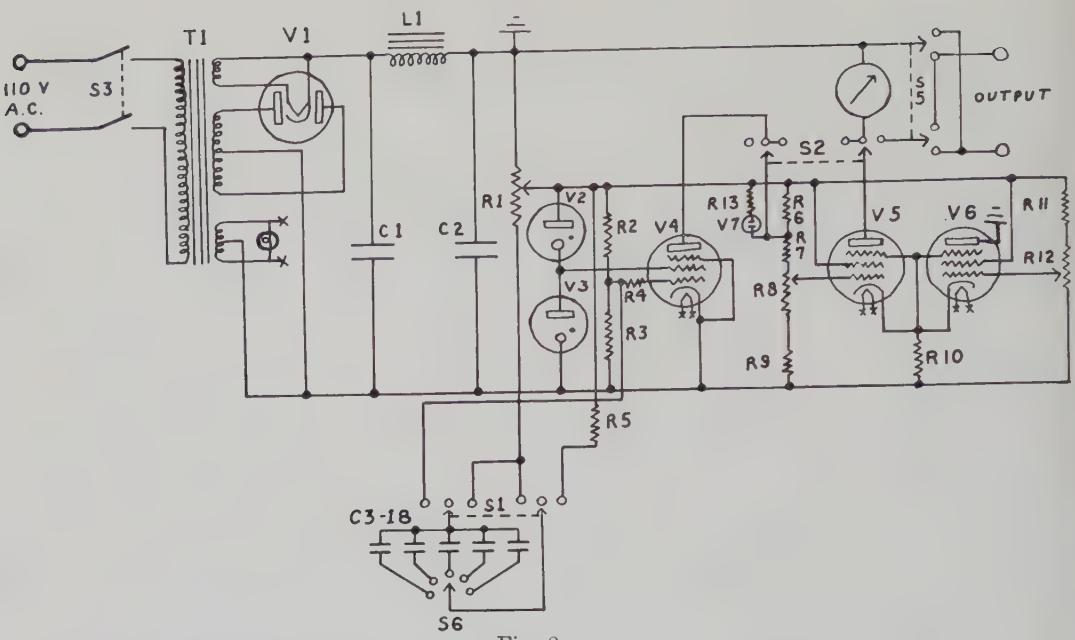

Fig. 2.

Schematic diagram of stimulator. The following components are required:

T1—Transformer, 800, 5.0, and 6.3 volts.
L1—Choke, 12 henrys at 100 ma.
C1, 2—8 fd. 600 valts.
C3 to 18—0.75, 0.5, 0.25, 0.1, .05, .025, .01, .005, .0025, .001, .0005, .00025, .0001, .00005, .000025, .00001 fd.
R1—20,000 ohms, 25 watts, voltage divider.
R2—6 megohms, ½ watt.
R3, 4—1 megohm, ½ watt.
R5—100,000 ohms, 1 watt.
R6—100,000 ohms, 5 watts.
R7—750,000 ohms, 1 watt.
R8—250,000 ohms, variable.
R9—80,000 ohms, 1 watt.
R10—1,500 ohms, 5 watts.
R11—750,000 ohms, 1 watt.
R12—500,000 ohms, variable.
R13—100,000 ohms, ½ watt.
V1—Rectifier, 5Z4.
V2—Voltage regulator, VR 150.
V3—Voltage regulator, VR 105.
V4—Pentode, 6SJ7.
V5, 6—Pentodes, 6F6.
V7—Miniature neon tube.
S1, 2—Ganged 2P3T switches.
S3—Power switch.
S5—2P2T reversing switch.
S6—1P16T selector switch.

sistance is kept below 15000 ohms. The duration of the stimulus may be varied in 16 steps from 0.02 to 1500 milliseconds, and its intensity accurately determined prior to administration by means of a conventional milliammeter. A switch provides for reversal of polarity. Thus it is possible to determine strength-duration curves, rheobase and chronaxia, relation between cathodal- and anodal-closing thresholds, and galvanic tetanus ratio.

The circuit, shown schematically in Fig. 2, is derived, with considerable modification, from a basic circuit described by Bauwens.[1] Automatic administration of shocks at fixed intervals was abandoned in favor of manual administration of single shocks, since this was found to be much better tolerated by human subjects. Grounding of the positive output terminal affords some protection from accidental shock.

The time during which the stimulating current flows is determined by the capacity chosen from the bank of condensers C3-18, controlled by switch S6. The function of the pentode V4 is to clip the wave form of the condenser discharge and to apply this voltage in an essentially square form to the grid of pentode V5, the amplitude of this voltage being controlled by the variable resistance R8. Because of the characteristics of the pentode V5, its plate current, which is also the stimulating current, is wholly dependent on its grid voltage so long as the plate voltage does not drop below about 100

[1] Bauwens, P., *Proc. Roy. Soc. Med.*, 1941, **34**, 715.

volts. This condition is satisfied so long as, with full output of 20 milliamperes, the output resistance does not exceed 15000 ohms, a condition easily met in practice.

By employing a common cathode resistance for tubes V5 and V6 their total plate current, and thus the load on the power supply, is kept constant whether a stimulus is being applied or not, eliminating the necessity for elaborate regulation of the power supply. Resistance R12 is adjusted so that with the intensity control R8 at maximum and switches S1 and S2, which are ganged, in the charging position, the milliammeter indicates 20 milliamperes. Thereafter R12 need not be adjusted further.

In operation, the duration of the stimulus is selected by S6, with S1-S2 in the center position to avoid sparking at the contacts of S6. Then S1-S2 is thrown to the charging position, charging the desired condenser. In this position the plate circuit of V4 is open, while the plate circuit of V5 is connected through the milliammeter. R8 is adjusted so that the meter indicates the desired intensity of stimulus, and then the stimulus is delivered by throwing S1-S2 to the stimulating position. This maneuver closes the plate circuit of V4, cutting off plate current in V5; then connects the plate of V5 through the subject rather than the milliammeter, and finally delivers the condenser discharge to the grid of V4, which clips it to square form and impresses it on the grid of V5, the resulting square wave of current in V5 being the stimulating current.

In practice, a large indifferent electrode is used, with the stigmatic electrode of 1.0 sq cm area placed over the nerve or motor point of the muscle to be tested. The threshold stimulus is then determined, first for currents of the longest duration available, and then for those of successively shorter duration until no further response can be obtained at maximum intensity. The values of threshold intensity so obtained are plotted against a logarithmic scale of duration, the result being a strength-duration curve. From this curve chronaxia may easily be determined. Galvanic tetanus ratio is arrived at by first establishing the threshold at which a simple muscle twitch is produced with a current of 1.5 seconds duration, and then increasing the intensity until a tetanic contraction persisting for the duration of the stimulus is secured. The ratio between these 2 intensities is the galvanic tetanus ratio.

Polarity of output is controlled by S3; the positive side remains at ground potential. Thus the relationship of anodal-closing to cathodal-closing thresholds may be easily obtained. In determination of galvanic tetanus ratio, observation of the small neon light, V7, which is extinguished during the passage of the stimulus, is helpful in determining the tetanus threshold, for which purpose it is mounted on flexible leads so that it may be observed at the same time as the contracting muscle.

Summary. A stimulator suitable for the determination of strength-duration curves, rheobase and chronaxia, ratio of cathodal to anodal thresholds, and galvanic tetanus ratio has been constructed of standard radio components. It has given reliable and reproducible results in cases of peripheral nerve injury.

15548

Effects of 2,4 - Dichlorophenoxyacetic Acid on Experimental Animals.*

Nancy L. R. Bucher. (Introduced by Joseph C. Aub.)

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2,4-Dichlorophenoxyacetic acid (2,4-D) is of biological interest because of its remarkable effects on plants. Its action is selective, not only with regard to different species of plants, but also with regard to the type of response in different plants and plant parts. It is a powerful growth stimulator in minute concentrations; actively growing plants are especially sensitive. Larger amounts produce morphologic changes which may occur at some distance from the point of application, and after an appreciable latent period, death of the plant ensues.[1-5]

In view of these properties it seemed worthwhile to investigate the effects of 2,4-D upon animals, with respect to toxicity, pharmacologic activity, and influence on normal and neoplastic growth. Plant hormones have previously been shown to inhibit the growth of transplantable tumors if applied directly to the tumor tissue,[6,7] but treatment of tumor-bearing animals by injection at a site remote from the tumor has been unsuccessful[7] except in rare instances.[8]

The sodium salt of 2,4-D was used in 1% solution in physiological saline, adjusted to approximate neutrality. Toxicity studies were carried out on young strain A male mice. The drug was injected either subcutaneously, intraperitoneally or intravenously and was found to be effective in the same general dose range by any of these routes. The drug has previously been reported to be nontoxic to animals and humans when administered orally.[4,5] In the present experiments the approximate LD_{50}, as determined by subcutaneous administration, was 280 mg per kg.

The injection of from 150 to 200 mg per kg at one time results in a myotonia-like syndrome that lasts from 8 to 24 hours or more in different animal species. The same signs have been produced in mice, rats, rabbits and dogs. Within a half to three-quarters of an hour following administration of the drug, the animal has ceased most spontaneous activity, and sits very still. However, he remains awake and alert. When he is now induced to make a sudden movement, such as a quick start, or a righting motion, spasm supervenes; his limbs spread out, and he falls and lies helpless, vainly trying to regain his footing. Opisthotonus may appear momentarily. The hind limbs are usually more noticeably affected than the fore limbs. If incited to continue his attempts to move, he will gradually recover. Motion is slow and awkward at first, but with continued effort approaches normal speed, smoothness, and control. After this, if the animal is allowed to remain still for 5 or 10 minutes, the initial syndrome recurs. In addition to the phenomenon of alleviation by exercise and exacerbation by rest, some animals exhibit a local hard knotty muscle spasm in response to a sharp percussive blow. Both features are characteristic of clinical myotonia. The diagnosis of myotonia has finally been established by myographic and electromyographic studies, now being done in conjunction with Dr. George Acheson of the Department of Pharmacology of the Harvard Medical School.

When larger doses (250 to 350 mg per kg)

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1 Zimmerman, P. W., and Hitchcock, A. E., *Contr. Boyce Thompson Inst.*, 1942, **12**, 321.

2 Hamner, C. L., and Tukey, H. B., *Bot. Gaz.*, 1944, **106**, 232.

3 Marth, P. C., and Mitchell, J. W., *Bot. Gaz.*, 1944, **106**, 224.

4 Hildebrand, E. M., *Science*, 1946, **103**, 465.

5 Mitchell, J. W., and Marth, P. C., *Bot. Gaz.*, 1944, **106**, 199.

6 Kline, B. E., Wasley, W. L., and Rusch, H. P., *Cancer Research*, 1942, **2**, 645.

7 Kline, B. E., and Rusch, H. P., *Cancer Research*, 1943, **3**, 702.

8 Tanaka, A., and Tuboi, S., *Gann*, 1940, **34**, 354.

are given to mice there is an initial period of myotonia as previously described, followed by rapidly increasing sluggishness and reluctance to move; tails are rigid; attempts at righting bring out a coarse clonic tremor. Inertia deepens into coma. The mice now lie on their backs with the 4 feet in the air. Their respirations are almost imperceptible. They are flaccid, and cold to touch. This state may terminate in death after several hours, or several days. In a few instances, after 8 to 12 hours, the mouse may go through the same stages in reverse order, finally recovering completely and without residua. Dosages of this magnitude have not been administered to other animals.

Other toxic effects of the drug which have been noted following parenteral administration are: (1) an irritant action on the eyes and nasal passages of dogs, resulting in sneezing spells, and violent rubbing of the eyes, which develops after a delay of 24 hours or more (2) gastro-intestinal disturbances in dogs manifested by vomiting and increasingly severe anorexia (3) the production of diarrhea in many, but not all mice.

Thus far no clear-cut chronic syndrome has been produced by continued administration of one or 2 daily injections to mice (1/3 to 1/5 of the LD_{50} per day for 3 weeks to 3 months).

Postmortem studies have not revealed any striking features peculiar to the drug. In acutely intoxicated mice the liver, kidneys and spleen grossly are dark and mottled. The upper part of the intestine is filled with a bile-colored liquid which is sometimes blood-stained. The bladder is full, and a mass of dry, hardened feces is caked about the anus. Microscopic examination shows wide dilatation of the blood vessels of lungs, liver and kidneys. In mice receiving prolonged treatment the only abnormalities noted have been attributable to infection. There was moderate atrophy of the liver in one chronically-treated dog, whose serum alkaline phosphatase rose from 5.0 to 39.4 Bodansky units in 3 weeks. This dog entirely refused food during the last week of life.

The peripheral blood of chronically-treated mice has not varied significantly from the controls with respect to hemoglobin, red cell, white cell and differential counts.

Small doses have not affected the growth rate of young mice. Larger amounts (1/4 of the LD_{50} daily) have retarded growth, probably through reduction of food intake. Mice who have survived this amount for 3 months are still alert, active and apparently normal in other respects. Repeated injections have not influenced the growth rates of 2 transplantable mouse sarcomas (Sarcoma 180 and Sarcoma 37) to any notable degree. Mitotic counts performed on these tumors at varying intervals after a single injection of 200 mg per kg did not differ significantly from the control values. Mice undergoing daily injections of 1/3 of the LD_{50} have become pregnant, and borne apparently normal litters at the end of a normal gestation period.

Summary. Parenterally-administered 2,4-D produces temporary myotonia in mice, rats, rabbits and dogs following a single injection. Repeated injections have failed to elicit either a characteristic chronic syndrome or a striking histologic picture; nor have they altered the process of reproduction and development in the dose ranges tested. The slight retardation of the growth rate of young mice on large daily doses is probably a manifestation of reduced food intake. Neoplastic growth has not been inhibited.

The author is grateful to Dr. Joseph C. Aub for the suggestion of this problem and for encouragement and advice. The technical help of Amory Glenn is also greatly appreciated.

15549

Effect of Some Atropine-like Drugs on Swing Sickness.

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This study was undertaken primarily to find remedies of value in the prevention of airsickness. Any drug to be of value in the treatment of airsickness should fulfill the following criteria; it should be effective immediately following oral administration; it should not impair the capacity of treated personnel to perform duties; it should not be toxic, habit forming, or cause disagreeable symptoms.

Methods. The drugs tested included atropine, scopolamine, hyoscyamine, homatropine, benzoyltropine, benzoyloscine, and pavatrine (B-diethylaminoethyl fluorene-9-carboxylate hydrochloride). All drugs or lactose placebos were contained in No. 1 pink capsules. The drug was given approximately one hour before swinging.

The swing test consisted of being swung in the sitting position through an arc of 150° on a swing with a radius of 14 feet from the center of the swing to the seat. The head was fixed with the plane passing through the lateral canthus of each eye and the external auditory meatus of each ear horizontal to the ground when the swing was at rest. Swinging was continued until the subject vomited or for a maximum of 20 minutes. Further details of the procedure have been described[1] in which it was shown also that there was a fair correlation between swing sickness and airsickness.

Drugs were studied in 3 ways: In the first, unselected subjects were swung and those that vomited within 20 minutes were used later for tests of the drug or placebo. In the second procedure, the subjects who vomited the first time were divided into 2 groups and before they were swung the second time half were given the drug and half placebos while the third time the order was reversed. In the third procedure, the subjects before having been swung were given either the drug or placebo and the incidence of vomiting in the various groups recorded.

The subjects were all either aviation cadets or aviation students. When the same subject was used repeatedly an interval of several days was allowed to elapse between swings and attempts were made to equalize the average interval between swings for the groups receiving drugs and groups receiving placebos. This was done because subjects swinging repeatedly with short intervals between may become accustomed to the motion.

The tests for side effects were chosen because they might reveal undesirable effects, such as dry mouth, interference with normal vision, etc. The subjects used for studies of these effects were selected without regard to susceptibility to swing sickness. Before being given a drug, each subject lay quietly for at least 5 minutes at which time his pulse rate and blood pressure were measured. Each man was given some paraffin to chew and told to salivate as much as possible into a small graduated cylinder during a 4-minute interval. The near point of accommodation was measured by means of a Prince rule. In this procedure, a piece of paper with a fine black line on it was first moved out from the eye until the line was no longer blurred and then from out to near the eye until the line appeared blurred. The mean of these 2 measurements was recorded. Each eye was tested separately. The drug or placebo was then given and 1½ hours later the observations were repeated.

Results. The results of the swing tests are given in Table I. They have been analyzed merely on the basis of the percentage of subjects in each group that vomited. Of the drugs tested scopolamine hydrobromide, 0.75

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† Now at Department of Physiol., University of Minnesota School of Medicine.

[1] Hemingway, A., submitted for publication.

TABLE I.
Swing Tests on Subjects After Atropine-like Drugs.

		Drug		Placebo			
	mg	No. Tested	No. Vomited	No. Tested	No. Vomited	Estimated protection %	P§
Scopolamine hydrobromide	0.5	21‡	5	21	9	44	0.18
" "	0.75	21*	5	21*	10	50	0.11
" "	0.75	21‡	9	21†	20	55	<0.01
Atropine sulfate	1.0	20‡	11	21†	20	42	<0.01
l-Hyoscyamine hydrobromide	1.0	20‡	7	21†	20	63	<0.01
Homatropine hydrobromide	12.0	20‡	14	21†	20	26	0.03
Benzoyltropine hydrochloride	50.0	20‡	14	21†	20	26	0.03
Benzoyloscine hydrochloride	50.0	20‡	13	21†	20	32	0.02
Demerol	100.0	8‡	7	21†	20	10	0.46
Pavatrine	250.0	20‡	11	21†	20	42	<0.01
"	250.0	52	18	365†	98	—	>1.00
Scopolamine hydrobromide	0.75	146	16	365†	98	59	<0.01
Atropine sulfate	1.0	106	15	365†	98	47	0.01

* Subjects acted as own controls, receiving the drug the second or third time swung and the placebo the other time.

† Those subjects receiving placebos were used at the same time several of the drugs were being studied and are grouped together.

‡ These subjects receiving drugs and the corresponding control group vomited on the initial selection swing.

§ Probability that observed difference could have occurred by chance.

TABLE II.
Parasympathetic Depressant Effects of Drugs.
Differences and standard deviations of differences between effects 1½ hours after administration of the drug and prior to administration.

Drug	No. subj.	Salivation		Pulse rate		Systolic blood pressure		Diastolic blood pressure		Accommodation	
		cc	S.D.	per min	S.D.	mm Hg	S.D.	mm Hg	S.D.	cm	S.D.
Placebos	58	1.0	1.8	— 9.6	6.0	—5.9	4.6	—2.8	5.8	—0.1	0.8
Scopolamine	20	—0.8	1.5	—15.4	4.0	—9.7	8.5	—3.0	6.5	0.4	1.4
l-hyoscyamine	20	—4.4	2.4	— 0.8	11.6	—5.2	5.6	—1.2	6.4	0.6	1.4
Pavatrine	23	0.5	2.6	— 9.9	4.7	—4.4	6.7	—0.9	4.6	—0.1	0.8
Atropine	20	—2.1	2.3	— 9.6	5.8	—4.8	8.2	—1.5	6.4	0.2	0.6
Benzoyltropine	21	—0.3	2.7	—10.8	4.0	—5.2	5.3	—0.1	4.6	0.3	1.0
Benzoyloscine	20	0.5	1.4	— 9.2	6.3	—5.7	5.2	—0.4	4.9	—0.1	0.9
Homatropine	20	—0.3	2.4	—13.2	4.4	—6.4	7.4	—0.3	7.0	0.6	1.6

mg, and atropine sulfate, 1.0 mg, were effective by 2 methods. Hyoscyamine hydrobromide, 1.0 mg, was effective by the only method it was studied. (The probability of the difference occurring by chance was less than 1 in 100). Homatropine, benzoyltropine and benzoyloscine were also moderately effective (the probability of the difference occurring by chance was less than 1 in 20). The effectiveness of Demerol was negligible and in some subjects caused nausea before they were swung. The study on Pavatrine in unselected subjects failed to confirm the earlier work on selected subjects and the incidence of vomiting after Pavatrine was even slightly higher than after lactose. There were no obvious factors to account for this difference. From the calculation of the estimated protection, it is obvious that not all of the drugs were of equal value but due to the limited number of subjects it was not possible to determine whether or not statistically valid difference in effectiveness existed. In general it may be said that scopolamine, atropine, and *l*-hyoscyamine were effective whereas homatropine, benzoyltropine and benzoyloscine were of a lower order of usefulness.

With the exception of Pavatrine, comparable results were obtained by the methods

when the subjects were selected and when they were unselected. This is reflected in the estimated protection of 50% and 55% with scopolamine in selected subjects and 59% in unselected subjects and in the 42% estimated protection with atropine in selected subjects compared with 47% in unselected subjects.

The effects of these drugs and some others on salivation, pulse rate, blood pressure and accommodation are presented in Table II. As was to be expected the resting subjects showed a slight decrease in blood pressure whether receiving the drug or placebo but there were no significant differences. Because of the biphasic action of the atropine-like drugs in first stimulating and then decreasing the effects, it was not surprising that there was considerable variation in the pulse rate. The effects were more pronounced for hyoscyamine where in some measurements the pulse rate taken 45 minutes after the drug was given revealed a mean decrease but after 1½ hours the mean rate was significantly higher than in the placebo group (probability of no difference less than 0.01). In the doses used, scopolamine and homatropine produced significantly greater decreases in pulse rate than the placebo. In all cases the probability of no difference was less than 0.01. Significant decreases in salivary flow (probability of no difference less than 0.01) were produced by hyoscyamine, atropine and scopolamine. The effect produced by hyoscyamine was greater than that produced by any other drug tested and produced a definitely dryer mouth than scopolamine alone (probability of no difference less than 0.01).

Discussion. Not enough compounds have been tested to show clearly the relation between chemical structure and effectiveness in motion sickness. From the limited data it appears that substitution of the tropic acid group, as it appears in atropine, hyoscyamine and scopolamine with a mandelic acid group, as it occurs in homatropine or a benzoic acid group as it occurs in benzoyltropine and benzoyloscine, results in a great loss of activity.

In general, there is a rough correlation between the inhibition of salivation and the effectiveness in motion sickness. That is, scopolamine, hyoscyamine and atropine all depress salivation appreciably in the doses employed; whereas, the other drugs tested do not produce appreciable decreases nor are they significantly effective in motion sickness. The effects of these drugs on respiration were not studied but it would appear from earlier work that in general, atropine, scopolamine and hyoscyamine are more effective in stimulating respiration than the other drugs that were studied. Although this correlation between salivary depression and effectiveness in swing sickness may be of some value it should be pointed out that the effect on salivation of scopolamine is significantly less than that of hyoscyamine whereas there was no suggestion that hyoscyamine was more than slightly superior to scopolamine in swing sickness in the doses employed.

Summary. Scopolamine, atropine, hyoscyamine are effective remedies in preventing motion sickness due to swinging whereas homatropine, benzoyltropine and benzoyloscine were less effective and demerol and pavatrine were of doubtful value. None of the remedies, in the doses employed, produced effects on the pulse rate, blood pressure or near point of accommodation. Significant decreases in salivary flow were produced by atropine, hyoscyamine and scopolamine.

15550

Effect of Various Substances on Swing Sickness.

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The substances selected for study were chosen for the following reasons: The sodium barbital, sodium ethyl-allyl-thiobarbiturate (V-5),[2] Army Motion Sickness Preventive and thiamine had been suggested as possible remedies for motion sickness; the combinations of scopolamine and ethyl-B-methyl-allyl-thiobarbituric acid (V-12)[2] and of scopolamine, chlorobutanol and benzedrine had been suggested as mixtures of greater effectiveness than scopolamine alone; the mixture of scopolamine and neostigmine might retain the effectiveness in swing sickness of scopolamine[1] without the undesirable side effects;[1] the pyridoxine because it has been reported to be of value in the vomiting of pregnancy[3] and of irradiation sickness;[4] the benzedrine because it has sometimes been employed with the barbiturates to overcome the central depressant effects; and the sulfadiazine because it had been suggested as a mass prophylactic for the prevention of respiratory infections and information was desired as to whether or not it increased susceptibility to motion sickness.

The Army Motion Sickness Preventive consists of 65 mg sodium amytal, 0.40 mg atropine sulfate and 0.16 mg scopolamine hydrobromide per capsule. In this study the equivalent of 2 capsules of the Army Motion Sickness Preventive was used as a single dose.

Sulfadiazine was given to a group of subjects every day for 7 weeks. The subjects were tested at the end of the 4th and at the end of the 7th week. A similar group received placebos for a similar period of time. All remedies except the sulfadiazine were given in No. 1 pink gelatin capsules, approximately one to 2 hours before swinging began. The method of testing susceptibility of subjects to swing sickness is the same as in the previous report.[1]

The results are shown in Table I. The sodium barbital, benzedrine, V-5, thiamine and pyridoxine were of negligible effectiveness. The results with pyridoxine are not inconsistent with its possible value in the nausea of pregnancy and of irradiation sickness since the latter may be associated with liver damage. The Army Motion Sickness Preventive is moderately effective as might be predicted from its content of atropine and of scopolamine.[1] The number of subjects employed with sulfadiazine is so small that the suggested effectiveness is of no statistical significance. The data do suggest, however, that sulfadiazine in this dose does not appreciably increase the susceptibility to motion sickness. Inconsistent results were obtained with the mixture of scopolamine and neostigmine. In the group of selected subjects the mixture was considerably more effective than in the group of unselected subjects. The explanation for this is not apparent. Perhaps of more importance, however, is the observation that the mixture seems to retain some of the motion sickness preventive value of the scopolamine alone. A study of the effects of this mixture on salivation conducted as in the previous report showed that the neostigmine had abolished almost entirely the depressant effect on salivation. The mixture of scopolamine and V-12 is no more effective than the scopolamine alone was found to be.[1] The addition of chlorobutanol and benzedrine to scopolamine gave a remedy that was highly effective but the observed superiority of this mixture over scopolamine alone was not statistically signi-

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1 Smith, P. K., and Hemingway, A., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 206.

2 Noble, R. L., *Proc. Assn. Comm. Army Med. Res., N.R.C. Canada*, 1943.

3 Willis, R. S., Winn, W. W., Morris, A. T., Newson, A. A., and Massey, W. E., *Am. J. Obs. Gyn.*, 1942, **44**, 265.

4 Maxfield, J. R., Jr., McIlwain, A. J., and Robertson, J. E., *Radiology*, 1943, **41**, 383.

TABLE I.
Swing Tests on Subjects After Various Substances.

		Drug		Placebo			
	mg	No. Tested	No. Vomited	No. Tested	No. Vomited	Estimated protection %	P§
Barbital, sodium	325	20	12	20	9	—	1.00
Benzedrine sulfate	10	20	13	20*	14	7	0.73
V-5	250	21	13	21*	15	13	0.52
Thiamine chloride	10	20	12	20*	14	14	0.66
USAMSP		19	11	19*	16	31	0.07
Sulfadiazine, 1 g†		19	4	18	4	0	1.00
Sulfadiazine, 1 g‡		19	5	17	7	36	0.33
Hyoscine hydrobromide	0.75						
prostigmine bromide	15.0	20	10	21	20	48	0.01
Hyoscine hydrobromide	0.75						
prostigmine bromide	15.0	81	17	365	98	22	0.28
Pyridoxine hydrochloride	100	33	15	82	30	—	1.00
Pyridoxine hydrochloride	200	28	9	82	30	12	0.66
Hyoscine hydrobromide	0.75						
V-12	250	104	14	365	98	50	0.01
Hyoscine hydrobromide	0.75						
chlorbutanol	500						
benzedrine sulfate	10	145	14	365	98	64	0.01

* Subjects acted as own controls, receiving the drug the second or third time swung and the placebo the other time.

† Tested after receiving the drug for 4 weeks.

‡ Tested after receiving the drug for 7 weeks.

§ Probability that observed difference could have occurred by chance.

ficant.

Summary. 1. Sodium barbital, benzedrine, V-5, thiamine, and pyridoxine were of no demonstrated value in the prevention of swing sickness. 2. Sulfadiazine in daily doses of 1 g did not appreciably affect the susceptibility to swing sickness. 3. A mixture of sodium amytal, atropine, and scopolamine was effective in swing sickness but probably not significantly more effective than could be predicted from its content of atropine and scopolamine. 4. The addition of neostigmine to scopolamine decreased the effectiveness of this mixture over that of scopolamine alone but did not entirely abolish it, although it effectively prevented the depressant action of scopolamine on salivation. 5. The addition of the thiobarbiturate V-12 to scopolamine did not increase its effectiveness in swing sickness. 6. A mixture of scopolamine, chlorobutanol and benzedrine was insignificantly more effective than the scopolamine alone in a similar dose.

15551

Effect of Anoxic Anoxia on Body Weight Loss in Rats.

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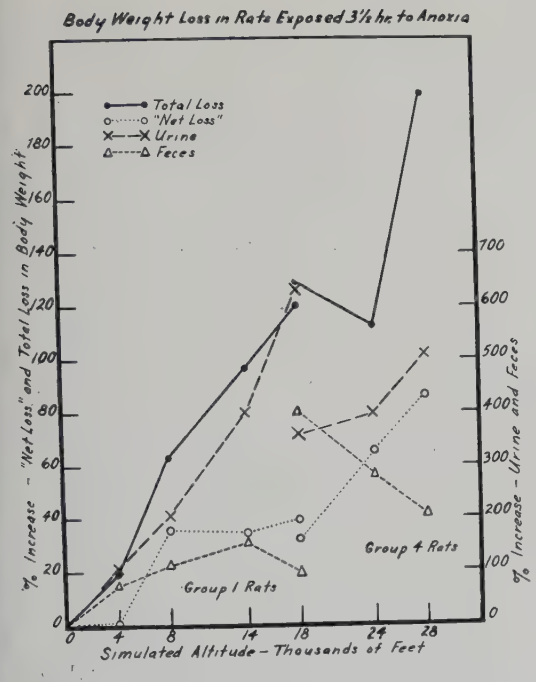
Fasting rats lose a significantly greater amount of weight when exposed to simulated altitudes of 8,000, 18,000 and 28,000 ft. than at normal barometric pressure.[1,2] Since the effect of anoxia on the weight and the water

[1] Van Liere, E. J., *Anoxia, Its Effect on the Body*, The University of Chicago Press, Chicago, 1942.

[2] Swann, H. G., and Collings, W. D., *J. Aviation Med.*, 1943, **14**, 114.



content of various organs is being studied by various workers, it seemed that information concerning total body weight losses occurring throughout the range of anoxia compatible with life would be helpful. An analysis of this weight loss into its components: urine, fecal and insensible water loss as Swann and Collings have made, is quite instructive, so this was done also. Furthermore, this procedure reveals the effect of anoxia on the secretion of urine, a problem which is not yet fully understood.

Procedure. Separately weighed rats were placed in individual metabolism cages in a decompression chamber for periods of 3½ hours; temperature and humidity within the chamber were determined. At the end of this period, the rats were weighed and the weight of feces and the volume of urine were determined.

The 6 rats constituting Group 1 were 4 months old (average wt 291 g) and were maintained on Purina Dog Chow. They were exposed to simulated high altitudes not oftener than twice a week and during a 5-month period received exposure to altitudes of 4,000, 8,000, 14,000, and 18,000 ft. Between exposures to altitude, control experiments were carried out with all factors the same except that the pressure was not reduced. The 6 rats in Group 4 were 3 months old (average wt 248 g) and were kept on Rockland Rat Diet—Complete. During the course of 6 months and in similar experiments these rats were exposed to 18,000, 24,000 and 32,000 ft. Before the final 4 experiments at 28,000 ft. they each received 1 mg cocaine hydrochloride intraperitoneally.

Results and Discussion. In the figure, the results are expressed as per cent change from the control at each altitude. In addition to the changes in total weight loss, urine secretion and fecal excretion; changes in "net loss" are indicated. "Net loss" is the difference between the sum of the weights of feces and urine (specific gravity assumed: 1.0) and the total body weight loss. This value actually includes losses by evaporation from voided urine and feces as well as from the lungs and skin. But since the error due to urine and fecal evaporation is controlled when experimental values are compared with control values, the changes in "net loss" must represent fairly well those in insensible water loss. The same argument is applicable to changes in urine and feces.

The average temperature in all determinations varied between 24.3° and 25.1°C. The greatest difference in average relative humidity between control and experimental determinations (range of differences: 1.5-17.0) was at 24,000 ft. where total loss is out of line, but in the opposite direction expected. Apparently other factors than those we attempted to control were of more importance.

The average body weight loss in 16 control determinations on the rats of Group 1 was 13.9 mg/g body weight; of this 2.7 mg/g was urine; 2.3 mg/g was feces and 8.9 mg/g was "net loss." Similar results were found with the rats of Group 4.

The threshold altitude for each variable was found by determining which differences between the control and experimental values were statistically significant by the method of Fisher.[3] The thresholds for increases in

[3] Fisher, R. A., *Statistical Methods for Research Workers*, fourth edition, Oliver and Boyd, London, 1932.

total loss, "net loss" and feces were between 4,000 and 8,000 ft.; for urine below 4,000 ft.

The curves for per cent increase in total weight loss and "net loss" for the 2 groups of rats are fairly consistent with each other and together indicate increases proportionate to the altitude. The rats of Group 4 did not display as intense a polyuria in response to anoxia as those of Group 1, but they, nevertheless, had an increase in urine secretion which was proportionate to the altitude. The rats of Group 4 had a greater increase in fecal excretion at all altitudes than those of Group 1. These differences between the 2 groups were most probably due to the character of the diet; that for Group 1 being inferior. The eventual decline of the fecal excretion curves with altitude was probably due to a reduced intake of the diet, since loss of appetite is a well known symptom of anoxia.[1] The increased fecal excretion confirms the impression gained in this laboratory that in dogs, also, defecation is frequently one of the responses seen at 28,000 ft. It is of interest that colonic motility as registered by an enterograph in barbitalized dogs is usually greatly reduced by anoxia.[4]

Swann and Collings,[2] who subjected their rats under different conditions to 18,000 ft. for periods of time between 6 and 23 hours, found much greater increases in the rate of insensible water loss than reported here. This may explain in part their failure to find a diuresis at high altitude. Time may be a factor also, because their results for an exposure of 23 hours were less than those at shorter exposures and practically no different than the controls. Their finding an increase in the rate of fecal loss during the 6-hour exposure only, is to be expected in fasting rats.

The increases, here reported, in urine secretion are even greater than those found by Silvette.[5] The few experiments in which cocaine was administered did not reveal increased urine secretion above that seen without this epinephrine-potentiating agent, nor did they reveal a reduced secretion. This result was obtained in spite of the fact that cocaine caused in the control experiments, an increase of over 200% in urine secretion. Such results are not favorable to the view that anoxia-induced changes in urine secretion are a result alone of epinephrine and/or augmented sympathetic impulses to the kidneys. Such an explanation is more in accord with the results found in anesthetized animals[6] where oliguria is frequently found as well as polyuria in response to anoxia. Furthermore, 32,000 ft. proved lethal to all but one of the rats, but in spite of this severe stimulation of the autonomic nervous system, they continued to respond with polyuria.

Summary. The body weight loss in rats is proportional to the altitude up to 28,000 ft. The thresholds for increased total body weight loss, "net loss" (insensible water loss) and fecal excretion lie between 4,000 and 8,000 ft.; that for urine, below 4,000 ft. The administration of cocaine did not change the urinary response to anoxia (28,000 ft.)

[4] Van Liere, E. J., Northup, D. W., Stickney, J. C., and Emerson, G. A., *Am. J. Physiol.*, 1943, **140**, 119.

[5] Silvette, H., *Am. J. Physiol.*, 1943, **140**, 374.

[6] Toth, L. A., *Am. J. Physiol.*, 1940, **129**, 532.

15552

Neural Effects of DDT Poisoning in Cats.

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The literature of the last few years contains several reports[1,2] of nervous disorders produced in laboratory animals by DDT poisoning. Our aim was to investigate whether DDT has, to any extent, a selective action

[1] Lillie, R. D., and Smith, M. I., *Pub. Health Rep.*, 1944, **59**, 979.

[2] Nelson, A. A., Draize, J. H., Woodard, G., Fitzhugh, O. G., Smith, R. B., Jr., and Calvery, H. O., *Pub. Health Rep.*, 1944, **59**, 1009.

TABLE I.

Animal No.	Wt, kg	Total amt DDT in mg/kg	Days injections given	Onset and duration of symptoms
263	2.1	370	1, 8, 11, 18, 28	Onset: 30th day Death: 33rd "
264	1.6	665	1, 8, 18, 28, 33, 39, 78, 85, 92	Onset: 232nd " Death: 235th "
287	1.5	200	1, 8	Onset: 13th " Death: 16th "
288	2.4	275	1, 31, 38, 45	Onset: 114th " Death 116th "
289	2.0	100	1	Died 3rd day without neurological signs
291	4.0	135	1, 13, 23	Onset: 42nd day Death: 45th "
292	2.2	225	1, 13, 23	Onset: 29th " Death: 29th "

on definite portions of the nervous system, such as other drugs possess.

Seven normal adult cats were given intramuscular injections of DDT solution in olive oil. This way was chosen with the belief that it afforded, as compared to the oral route, a better control of dosage and a prolonged absorption time. By slower absorption, we hoped to produce less liver damage, and to be able to observe more easily the effects on the nervous system. It was hoped to obtain *chronic animals,* but, even though the dosage was far below the lethal amount, the animals either quickly developed steadily increasing signs of intoxication, ending in death, or, after transient illness, recovered completely.

The data gathered from our experiment are summarized in Table I. The occurrence of transient symptoms is not represented.

When using the same amount for each individual injection, and the same interval between injections, we observed in our animals a great variability of response. One animal died after only 135 mg per kg, in 3 injections, while another remained in good condition after having received a total dose of 665 mg/kg.

Very often, if not always, when the first signs of neurological disorder appeared, the animals exhibited signs resembling those of a cold: congestion and hypersecretion of the eyes and nose, without any rise in temperature. We could not decide whether these were autonomic effects of intoxication, the manifestation of a latent infection brought to light by the decreased resistance of the animals, or whether the occurrence of the neurological disorders was promoted by an occasional infection. This seems unlikely, since in our animals these symptoms resembling a cold and the neurological disorders always occurred simultaneously.

A few days (between 3 and 6) after the last injection, the animals began to show definite stiffness, especially in the hind limbs. Very soon afterward a fine tremor developed, first seen only in certain postures and movements, but rapidly becoming more coarse and permanent. If the animals recovered, after a few days (usually less than a week), the symptoms progressively disappeared and never went any further than this tremor.

In other cases, after 2 or 3 days, the tremor increased in amplitude and became constant, while the stiffness extended to the proximal muscles of the limbs and to the trunk. At that period the animal continued to eat well and was still able to walk. The only sensory change noticed was obvious hyperesthesia. After that the clinical picture changed very rapidly. Muscular twitching appeared, first in the face, and soon became generalized. The animal could not walk, but retained consciousness. Within one day, the twitching became grosser, with resultant clonic movements of the entire limb. Finally the condition resembled status epilepticus, and the animal died. Injections of barbiturates may prevent the clonic movements for a short

time.

Pathology. The lesions were the same in all animals with progressive symptoms, whether they died spontaneously or were killed by exsanguination. They consisted in diffuse degeneration of the ganglion cells throughout the brain, without any visible difference between different areas. In Nissl sections, the ganglion cells showed either vacuolar degeneration, with swelling of the cell, disappearance of Nissl bodies and increased colorability of the dendrites, or pyknosis. Only occasional, normal cells were found. In Weigert-Pal sections, no definite changes could be found.

Clusters of unidentified cells were observed close to the ependymal lining. Such clusters have been described in various species. We are unable to decide whether these clusters have any pathological significance. No alterations were found in the liver, aside from moderate hyperemia of the capillaries. In none of our cases was there any fatty degeneration, such as has been described as one of the most constant features of DDT poisoning after ingestion of the drug.

No alterations were observed in other organs.

Conclusion. Progressive neurological symptoms were produced in cats by the intramuscular injection of DDT. The signs evolved in the sequence of stiffness, tremor, clonic movements, and death.

An animal with chronic neurological symptoms was not obtained, either because death supervened or because the animal returned toward normality.

Nissl sections revealed diffuse damage to the ganglion cells of the brain, characterized by vacuolar degeneration or pyknosis. No alterations, other than capillary dilatation, were recognized in the liver.

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Some Effects of Depletion and Repletion in Proteins on Body Fluids in Adult Dogs.*

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A decrease in plasma volume and a nutritional edema accompanies hypoproteinemia in dogs.[1-3] The fall in concentration of plasma proteins is associated also with an increase in the utilization of nitrogen, the nitrogen balance indexes of proteins being greater in protein-depleted than in normal animals.[4] An analysis of these data suggests that there are regular shifts in body fluids and in the utilization of nitrogen as the protein stores of the animal are altered. Experiments were organized, therefore, to study changes in plasma and "available fluid" volumes and in the excretion of various forms of nitrogen in adult dogs during control, depletion, and repletion periods.

Methods. Four dogs were depleted by feeding a protein-free diet; 3 days of plasmapheresis being used initially to speed up the process of depletion. The details of this method of

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The subject matter of this paper has been undertaken, in part, in cooperation with the Quartermaster Corps Committee on Food Research.

1 Allison, J. B., Anderson, J. A., and Seeley, R. D., *Bull. N. Y. Acad. Sci.*, in press.

2 Weech, A. A., Goettsch, E., and Reeves, E. B., *J. Exp. Med.*, 1935, **61**, 299.

3 Weech, A. A., Wollstein, M., and Goettsch, E., *J. Clin. Inv.*, 1937, **16**, 719.

4 Allison, J. B., Seeley, R. D., Brown, J. H., and Anderson, J. A., *J. Nutrition*, 1946, **31**, 237.

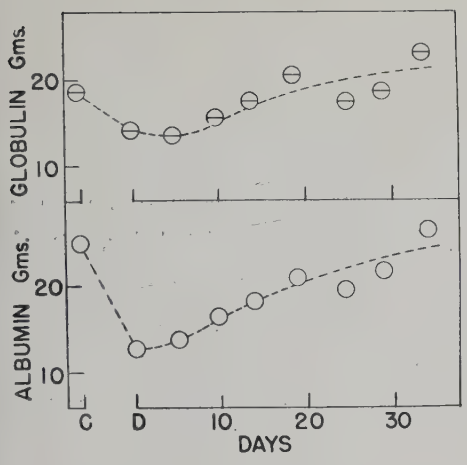

FIG. 1.

Total circulating plasma albumin and globulins in dog No. 68 before (C) and after depletion in proteins (D) and during 35 days of feeding 0.35 g of casein hydrolysate nitrogen/day/kilo of body weight.

depletion have been described previously.[5] During the repletion period 2 dogs were fed orally a casein hydrolysate, and 2 were fed a lactalbumin hydrolysate,† each dog receiving 0.35 g of nitrogen/day/kg of body weight. The plasma and "available fluid" volumes were determined according to the technics described by Gregersen and Stewart.[6] Nitrogen determinations on the blood and urine were made using the Pregl Micro-Kjeldahl. The albumin and globulin fractions were determined by the salt fractionation method of Howe.[7]

Urine ammonia and urea nitrogen were determined by the aeration method of Van Slyke and Cullen,[8] creatinine and creatine were determined by the alkaline picrate procedure of Folin,[9] and uric acid was analyzed by the indirect method of Folin.[10] Urine alantoin was determined by the procedure of Young and Conway[11] and the ninhydrin-carbon dioxide method of Van Slyke, MacFadyen and Hamilton[12] was used to determine the α-amino nitrogen.

Results. The data in Fig. 1 illustrate the

TABLE I.

Data Obtained on 4 Dogs Before and After Depletion in Proteins and After Repletion from Feeding 0.35 g of Nitrogen per Kilo of Body Weight for 30 Days. Dog 28 and 65 received a lactalbumin hydrolysate and Dogs 42 and 68 a casein hydrolysate during the 30-day repletion period.

Dog No.	Wt, kg	Plasma protein, g %	Plasma vol., (P) ml	Available fluid (A) ml	A/P
		Control.			
28	10.5	6.75	444	3100	7.0
65	10.8	7.14	475	3000	6.3
42	12.1	6.90	430	2600	6.0
68	12.0	7.04	625	3500	5.6
		Depleted.			
28	10.7	4.52	373	3500	9.3
65	11.1	4.50	410	4400	10.7
42	10.6	4.72	400	3600	9.0
68	11.1	5.20	502	4400	8.7
		Repleted.			
28	11.4	6.50	443	2800	6.3
65	11.7	6.51	626	3200	5.1
42	10.3	6.80	465	2600	5.6
68	11.4	7.07	600	3400	5.6

5 Seeley, R. D., *Am. J. Physiol.*, 1945, **144**, 369.

† These hydrolysates were prepared by Dr. Bacon F. Chow of the Squibb Institute for Medical Research. Details concerning these hydrolysates will be published elsewhere.

6 Gregersen, M. I., and Stewart, J. D., *Am. J. Physiol.*, 1939, **125**, 142.

7 Robinson, H. W., Price, J. W., and Hogden, C. G., *J. Biol. Chem.*, 1937, **120**, 481.

8 Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1916, **24**, 117.

9 Folin, O., *J. Biol. Chem.*, 1914, **17**, 469.

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11 Young, E. G., and Conway, C. F., *J. Biol. Chem.*, 1942, **142**, 839.

12 Van Slyke, D. D., MacFadyen, D. A., and Hamilton, P. B., *J. Biol. Chem.*, 1943, **150**, 251.

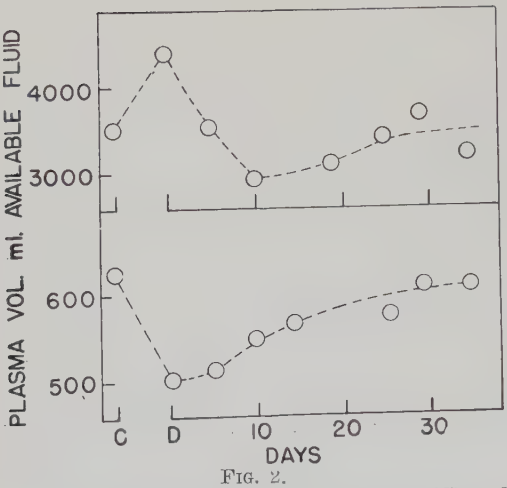

FIG. 2.

Available fluid and plasma volumes before (C) and after depletion in proteins (D) and after 35 days of feeding 0.35 g of casein hydrolysate nitrogen/day/kilo of body weight.

effect of depletion upon plasma proteins in one dog, typical of all 4. These data prove that plasma albumin decreases markedly while plasma globulins decrease slightly below control values in the depleted animal. The reduction in globulin fractions may represent a decrease in the protein stores of the lymphatic tissues. Certainly the α globulin fraction is not reduced, the reduction in globulins being restricted to the β and γ fractions.[13] Under these conditions, the dogs become very susceptible to disease, developing kennel sores and other skin disorders. Replenishment of protein stores by feeding a high-quality protein restores rapidly the natural resistance of the animal to these disturbances. Fig. 1 demonstrates the gradual return of albumin and globulin fractions from the depleted to the normal condition when the dog was fed a casein hydrolysate. Similar regenerations were found in the other 3 animals, one receiving the casein hydrolysate and the other 2 lactalbumin hydrolysate. A critical evaluation of the regeneration of the plasma proteins in these experiments has been made through the analysis of electrophoretic patterns which were obtained on these and a number of other dogs.[14]

[13] Chow, B. F., Allison, J. B., Cole, W. H., and Seeley, R. D., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 14.

The data in Table I record the effects of depletion and repletion in proteins on the plasma and available fluid volumes.

The plasma volume decreases below the control value in the depleted dog, returning toward normal during the repletion period. The available fluid, on the other hand, increases in the protein-depleted dog, returning to control levels upon repletion. Thus the ratio between the available fluid and plasma volumes increases in the depleted animals to above normal. This increased ratio detects a "nutritional edema" long before increased fluid in tissue spaces can be observed clinically.

The data in Fig. 2 illustrate graphically, in more detail than in Table I, the changes in available fluid and plasma volumes which accompany depletion and repletion in proteins. The rapid decrease of available fluid volume which occurred in all dogs soon after the repletion process started is especially noteworthy.

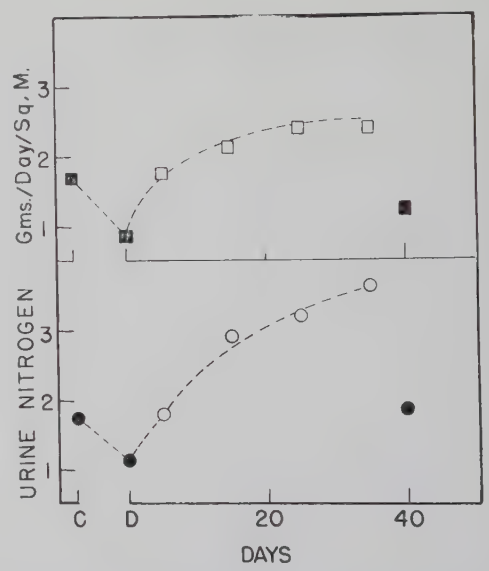

FIG. 3.

Total daily urine nitrogen excretion in dogs 28 □ and 68 ○ while on a protein-free diet before (C) and after depletion in proteins (D) and during 38 days of feeding 0.35 g of hydrolysate nitrogen/day/kilo of body weight. The last point at 40 days represents the daily excretion of nitrogen on a protein-free diet after repletion.

[14] Chow, B. F., Allison, J. B., Cole, W. H., and Seeley, R. D., in press.

TABLE II.

Daily Excretion of Compounds Containing Nitrogen During a 5-day Protein-free Feeding Period Before and After Depletion in Proteins and Repletion from Feeding for 35 Days 0.35 g of Hydrolysate Nitrogen per Day per Kilo of Body Weight.

Condition	Total N, g/day/sq.m.	Urea and Ammonia N, g/day/sq.m.	Creatine N, g/day/sq.m.	Creatinine N, g/day/sq m.	Uric acid N, g/day/sq.m.	Alantoin N, g/day/sq.m.	Amino N, g/day/sq.m.
			Lactalbumin Hydrolysate, Dog 28.				
Control	1.73	1.19	0.04	0.20	0.01	0.19	0.026
Depleted	0.84	0.46	0.02	0.13	0.01	0.19	0.031
Repleted	1.22	0.76	0.02	0.16	0.01	0.17	0.028
			Casein Hydrolysate, Dog 42.				
Control	1.35	0.84	0.03	0.17	0.01	0.24	—
Depleted	0.82	0.49	0.01	0.11	0.01	0.17	0.021
Repleted	1.60	1.06	0.04	0.14	0.01	0.20	0.022
			Casein Hydrolysate, Dog 68.				
Control	1.72	1.11	0.02	0.20	0.01	0.21	0.035
Depleted	1.13	0.70	0.02	0.16	0.02	0.16	0.025
Repleted	1.86	1.33	0.03	0.16	0.01	0.19	—

Fig. 3 illustrates data on urine nitrogen excretion obtained on 2 of the dogs, typical of all 4. The first point (C) records the urine nitrogen excretion of the normal dog while receiving the protein-free diet. The second point (D) records the excretion of urine nitrogen of the depleted dog while receiving the protein-free diet. The white squares and circles illustrate data obtained during the repletion process while dog No. 28 was receiving 0.35 g of lactalbumin hydrolysate nitrogen and dog No. 68 was receiving 0.35 g of casein hydrolysate nitrogen/day/kg body weight. The last black point records data obtained at 40 days, while the repleted dog was receiving a protein-free diet. These data prove that the excretion of body nitrogen on a protein-free diet is decreased below control values in the depleted dog. Upon repletion this excretion of body nitrogen returns toward control values, the body protein stores being replenished. The gradual increase in the excretion of nitrogen during the period of repletion is due to the gradual increase in body protein stores accompanied by a decrease in retention of dietary nitrogen. These data supplement those previously published where a reduction from control values in nitrogen excretion and an increase in nitrogen balance index of the dietary protein above normal was demonstrated in the depleted dog.[4]

Table II records the average daily urine excretion of compounds containing nitrogen during the protein-free feeding periods before and after depletion and after repletion in proteins.

These data prove that the decrease below control levels of urine nitrogen in the depleted dog is due primarily to a decrease in ammonia and urea nitrogen.

Summary. The plasma albumin decreases markedly while the total plasma globulins decrease slightly below control values in the protein-depleted animal. The dogs are very susceptible to disease in the depleted state, a susceptibility which is reduced upon repletion in proteins. The plasma volume drops and the available fluids increase as the total circulating plasma albumin is reduced by the process of depletion. This nutritional edema is corrected rapidly by repletion in plasma albumin.

The excretion of urine nitrogen gradually increases during the feeding of protein nitrogen in the repletion period. This gradual increase is the result of a decrease in the utilization of the protein and of an increase in the excretion of nitrogen from body stores. The decrease in urine nitrogen excretion below normal which occurs in the hypoproteinemic dog is primarily the result of a decrease in ammonia and urea nitrogen.

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Treatment of *Schistosomiasis mansoni* with Neostibosan.*

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Twelve patients with ova of *S. mansoni* in their stools were treated with the pentavalent antimonial drug, neostibosan (Winthrop). The patients were all males with ages ranging from 15 to 32 years. The drug was administered intravenously during a 2-week period of hospitalization. During the first 3 days, doses of 0.2, 0.6 and 0.9 respectively were usually given, and thereafter either 0.6 or 0.9 was generally injected every day until the end of hospitalization. All patients tolerated the drug well and no serious reactions were seen. A low grade fever and abdominal pain, headache, nausea or anorexia were observed in most of the patients.

The effect of the drug on the number of schistosome eggs passed in stools was carefully observed. Stool examinations were made daily before and during treatment and on as many daily specimens as possible for each follow-up examination. The technic used for the quantitative determination of eggs in feces has been described previously.[1]

The effect of treatment on the schistosome infections, as determined by the number of alive and dead schistosome ova in stools may be seen in Table I. Although the majority of the patients (9 out of 12 or 75%) had stools negative for live and dead ova one to 2 months after treatment, 8 out of 11 (73%) relapsed 3 and 4 months later. Three out of 11 patients, however, have remained negative throughout, for a period of 11 months.

Although the per cent of cases which has remained negative for ova of *S. mansoni* after

TABLE I.
Number of Live and Dead Schistosome Eggs in Patients Treated with Neostibosan.

Total drug administered	Eggs per ml of stool															
	Before treatment		Months after treatment													
			1		2		5		6		8		11			
	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead		
9.5	780	2,730			585	2,145			195	195			195	0		
8.6	780	3,900			195	780			1,365	390	1,170	1,050				
8.9	1,560	1,755			0	0			585	1,365	5,070	3,510	390	780		
8.6	7,020	3,900			2,340	1,950			390	585	1,170	1,365	1,170	488		
7.7	1,950	585			0	0			95	0	0	0	195	585		
9.5	1,560	1,365			0	0			0	195	0	0	195	195		
12.5	975	585	0	0	0	0	0	0			0	0	0	0		
11.6	195	2,340	0	0			0	0			0	0	0	0		
12.2	2,340	1,170	0	0							0	0	98	0		
12.5	1,755	585	0	0	0	95	390	195			0	98	0	0		
12.5	1,365	3,315	0	0	0	0	0	0			0	0	0	0		
8.9	390	195	0	0	0	390	0	95			0	98	0	0		

* The work described in this paper was done under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the School of Tropical Medicine, San Juan, P.R.

[1] Hernández-Morales, F., Suárez, Ramón M., Pratt, C. K., and Oliver-González, J., *Puerto Rico J. Pub. Health and Trop. Med.*, June, 1946.

treatment with neostibosan is small (3 out of 11 examined or 27.3%) there is evidence indicating that the drug has parasitotropic effects. This is also suggested by the fact than the egg count 11 months after treatment in 6 of the patients are appreciably lower than the counts before treatment. There is the possibility that in those patients in whom the infection was not eradicated insufficient drug was administered. Retreatment with neostibosan was found to be necessary by Culbertson, Rose and Oliver-González in humans infected with microfilaria of *W. bancrofti*.[2] It seems possible that the use of a greater amount of drug may produce a larger percentage of cures.

2 Culbertson, J. T., Rose, H. M., and Oliver-González, J., *Am. J. Hyg.*, 1946, **43**, 145.